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PHOTOPERIODICITY IN THE FEMALE FERRET

BY D. S. HART

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(*Received* 15 *February* 1950)

(With Nine Text-figures)

INTRODUCTION

Previous work has shown (Bissonnette, 1932) that added electric light after dusk has a pronounced stimulatory influence on the anterior pituitary (Hill & Parkes, 1933), causing the onset of oestrus in the ferret during the non-breeding season of the year from September to March. Marshall (1940) also showed that the time of the onset of the breeding season caused by exposure to light varied with the intensity of the light.

The fact that light promotes the onset of the oestrus in those mammals which have a summer breeding season has hitherto been assumed to be due to the effect of the light itself as the primary activating influence, and that darkness was merely an alternative to light. Thus the term 'light ration' has been frequently used. Any possibility that darkness as such has an influence on the function of the anterior pituitary and thus affects the oestrous cycle has been left uninvestigated.

In recent work with chrysanthemums Borthwick (1947) has shown that what are termed 'short-day' plants can be made to flower after treating them with a short light period and a long dark period during the 24 hr., whereas 'long-day' plants placed under the same treatment did not flower. This effect was due to the fact that in the 'short-day' plants the dark period in each 24 hr. was long, and not because the light period was short.

This raises the question whether the stimulating agent in summer breeding animals is the total amount of light per 24 hr. or whether it is the length of the dark period. To test this it was decided to set up experiments, using ferrets, in which the length of the dark period in relation to the length of the light period could be varied by using a different sequence of light and darkness over the 24 hr. period.

MATERIAL AND METHODS

The experiments were conducted in two series, the first extending over the winter non-breeding period (approximately September to March) of 1947–8, and the second during the same period of 1948–9.

1947–8 *experiments*. In these experiments treatment with artificial light was superimposed upon exposure to natural daylight. The length of the day was defined as that period between civil twilight a.m. and civil twilight p.m. as given in the *Nautical Almanac* and corrected for the latitude of Cambridge. Artificial light was obtained from Crompton 40 W. fluorescent tubes, daylight colour, with an efficiency of 40 lumens per watt and giving a light intensity of 22–24 f.c. on the floor of the pen when set at a height of 36 in. above it. This artificial light has much the same spectrum as daylight, except that it has no ultra-violet (see Fig. 1). The clockwork time switches which controlled the artificial light were adjusted every 3 days to conform to the requirements of the experiments.

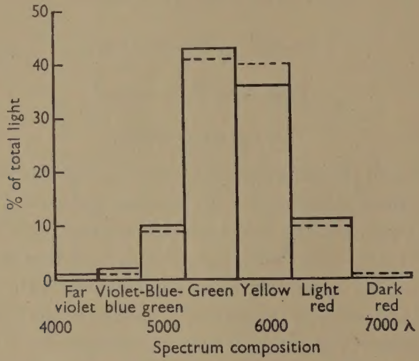

Fig. 1. Comparing spectrum of natural daylight with that of fluorescent light. ——— natural daylight level, - - - - - fluorescent light level.

Eight mature anoestrous female ferrets, which had not bred during the previous breeding season, were used; they had been in anoestrus for two and a half months when the experiments began on 5 December 1947. They were divided into four groups of two each. The groups were housed separately in adjacent rooms and thus any danger of one experiment being affected by the light from another was eliminated. Every three days the ferrets were examined for signs of the onset of oestrus, this being very clearly indicated by the gradual swelling of the vulva, until when in full oestrus the vulva is quite large. They were then mated with treated males and subsequently produced normal litters.

Details of the experiments were as follows. All four groups were exposed to natural daylight which decreased in duration day by day from 5 to 21 December and thereafter increased from 21 December until the end of the experiments (see Fig. 5).

Group I. One hour's exposure to artificial light was given about midnight, breaking the night into two equal periods of darkness (see Fig. 2).

Group II. As group I, but the duration of the artificial light was increased by half an hour every three days (see Fig. 3).

Group III. The periods of exposure to artificial light were of the same length as in group II but the artificial light was given at the end of the day and not in the middle of the night (see Fig. 4).

Group IV. Controls: no artificial light (see Fig. 5).

1948–9 *experiments.* For these experiments thirty female ferrets were bred at the Animal Research Station during the summer of 1948; by the time the experiment commenced on 30 October 1948, they were therefore about three to four months old and well grown, but virgin and in a completely anoestrous condition. At no time did any of them show the least evidence of oestrus up to the start of the experiment.

The ferrets were divided into three experimental groups of eight each, and one control group of six. The experimental groups, designated A, B and C, were housed in the same pens and in the same adjacent rooms as were used in the 1947–8 experiment. The only difference was that the rooms housing groups B and C had been made completely light-proof; the room housing group A was open to daylight at the start of the experiment but was rendered light-proof at a later stage. The artificial lighting had been retained from the previous year; the quality and intensity of the artificial light was therefore the same for both sets of experiments. The same time-switch circuits were also employed.

In order to identify individual ferrets a system of ear-marking by means of notches in one or both ears was used. Feeding of all groups was the same; the bulk ration consisting of bread and milk was supplied once daily and the pens were cleaned out at the same time, both these operations being made to coincide with a light period in the sequence. All ferrets were examined twice a week for changes in vulva size, in order to obtain an indication of the onset of oestrus or of anoestrus.

Group A were exposed to natural daylight (admitted through two windows facing south and south-west) and to continuous artificial light for the first eleven weeks of these experiments. After 11 weeks the room was rendered light-proof to exclude daylight and the animals were exposed to continuous artificial light for the remainder of the experiment.

Group B were subjected to a constant sequence of 8 hr. light–4 hr. darkness.

Group C were subjected to a constant sequence of 16 hr. light–8 hr. darkness.

After results had been obtained in the above-mentioned groups, that is, after all the animals had come into oestrus, new groups were constituted as follows.

Group A_1 (eight ferrets from group B); a constant sequence of 4 hr. light–20 hr. dark.

Group C_1 (eight ferrets from group C); a constant sequence of 8 hr. light–16 hr. dark.

After results had been obtained, that is, after all the animals had gone into anoestrus, a final reconstitution gave:

Group A_2 (four ferrets from group C_1 and three from group A_1); a constant sequence of 4 hr. light–2 hr. dark.

Group X (four ferrets from group C_1 and five from group A_1); a constant sequence of 8 hr. light–16 hr. dark.

The control group were exposed to natural daylight only, on a decreasing plane up to 21 December and thereafter on an increasing plane for the remaining period of their effectiveness as controls.

It should be noted that in the final reconstitution of groups, group A_2 was made up of four ferrets from group C_1 (previously brought into oestrus on a 16 hr. light–8 hr.

dark sequence and then brought into anoestrus on an 8 hr. light–16 hr. dark sequence) and three ferrets from group A_1 (previously brought into oestrus on an 8 hr. light–4 hr. dark sequence and then brought into anoestrus on a 4 hr. light–20 hr. dark sequence). This reconstruction was an attempt to balance out any residual effects of previous treatment, such as have been observed by Hammond Jr. (1949) in ferrets and mink. The remaining ferrets of groups C_1 and A_1, now group X, continued on the 8 hr. light–16 hr. dark sequence as semi-controls to group A_2.

RESULTS

1947–8 *experiments*. All the experimental groups came into full oestrus, the first animal on 25 January 1948, after a latent period of 50 days, and the last animal on 9 February 1948, after a latent period of 67 days. The control group remained in anoestrus throughout, and on 1 March 1948, after a latent period of 87 days, was still showing no signs whatever of approaching oestrus. Ultimately the first control animal came into oestrus on 30 March and others followed in a few days, the latent period being 117 days. Fig. 6 shows the time relationships of the various groups.

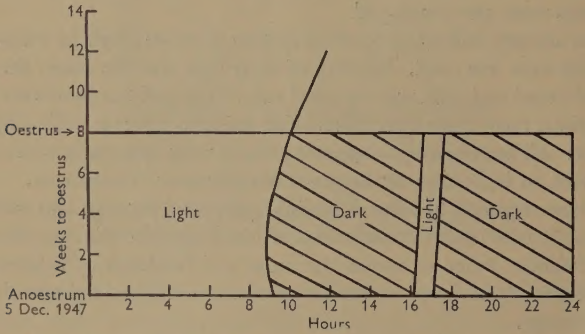

Fig. 2. Group I. Light rhythm chart showing solar increase only.

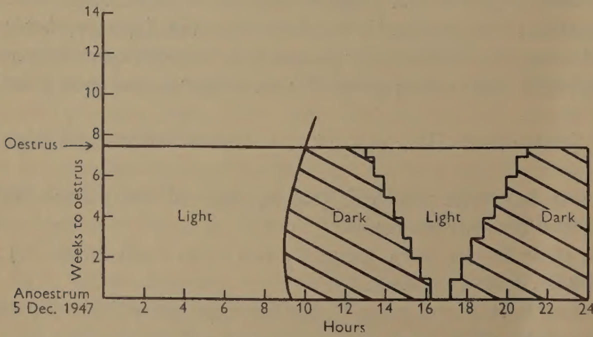

Fig. 3. Group II. Light rhythm chart showing both solar and artificial increase rates.

There can be no doubt that considerable acceleration of the onset of oestrus had been brought about in the three experimental groups by exposure to artificial light in addition to daylight.

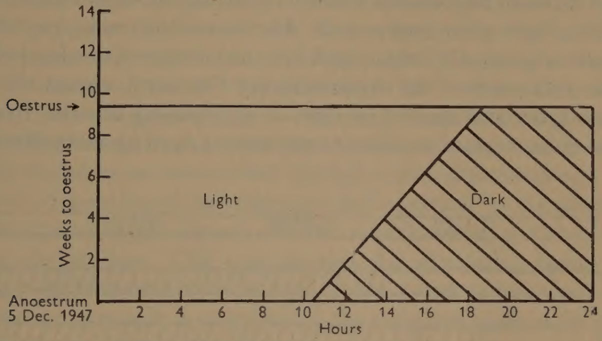

Fig. 4. Group III. Light rhythm chart showing artificial increase rate.

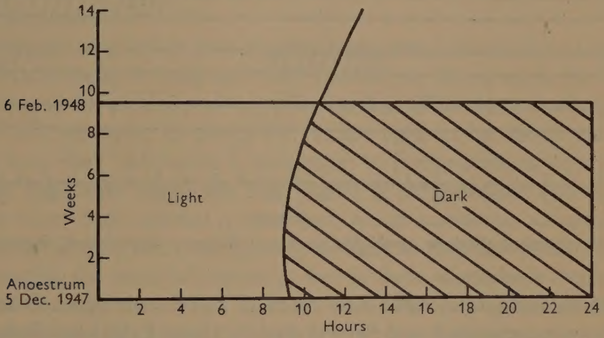

Fig. 5. Group IV (controls). Light rhythm chart showing solar increase only.

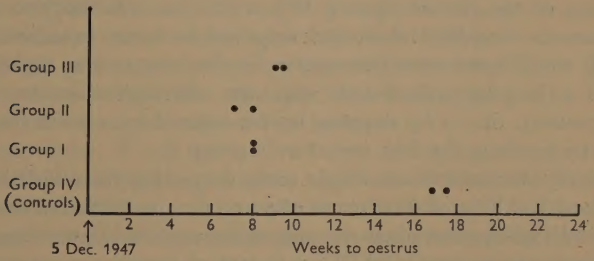

Fig. 6. Scatter plot showing relationship of oestrus onset in all animals.

The total amount of artificial light per 24 hr. period, however, varied considerably between group I and the other two experimental groups, which had approximately the same total amount of light per 24 hr. period. Group I animals were receiving a total of only 11⅓ hr. light per 24 hr. when they came into oestrus, whilst groups II and III had reached totals of 18 and 18¾ hr. light per 24 hr. respectively when they came into oestrus. This seems to indicate that the total quantity of light given in any 24 hr. period is not so important a factor in inducing the onset of oestrus as is the sequence of long-light: short-dark periods. Further evidence in support of this comes from the controls (group IV) whose total light had increased to a level of 11 hr. per 24 hr. by the 10th week of the experiment (13 February), almost the same total light as group I, but still showed no signs of approaching oestrus. In actual fact, these controls did not come into oestrus until about 1 April (9 weeks after the group I animals), when their total light had reached 14 hr. per 24 hr. The important difference between groups I and IV was that in group I the long-dark period was divided into two equal periods by 1 hr. of light, and in this way the short-light: long-dark sequence of the controls (group IV), which was inhibitory to oestrus, was converted into the long-light: short-dark sequence necessary to induce oestrus.

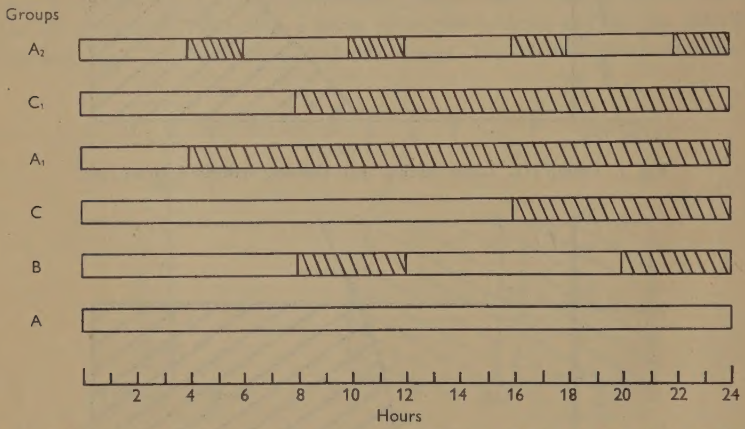

Fig. 7. Light treatment rhythms, unshaded portion indicates a light period, shaded a dark one.

Groups II and III also came into oestrus for the same reason, namely the establishment of a long-light: short-dark sequence, although this was achieved in a different manner, that is by stepping up the rates of increase in the light ration rather than by breaking the dark period as in group I.

The gradually increasing plane of light or the decreasing plane of darkness appears in itself to have had little or no material effect on the production of oestrus in these animals, a result which contradicts the suggestions made by Yeates (1949) for sheep. Groups I (broken dark period) and IV (controls) both had the same rate of increasing daylight throughout, i.e. the natural rate of increase, and group I came into oestrus whilst group IV did not. Groups II and III both had an artificially stepped up rate of increasing light, greatly in excess of group I, yet as compared with group I, they showed no significant acceleration of the onset of oestrus. In point of fact group II was a few days ahead and group III a few days behind group I. If an increasing or decreasing plane of light was significant, it should have been shown by differences between groups II and III on the one hand and group I on the other.

The consensus of opinion has hitherto tended towards the idea that the long-light period (or 'light ration') was the most likely stimulating factor. This does not necessarily appear to be the case and perhaps equal emphasis at least should be placed on the short-dark period. Yeates (1949), in his very extensive survey of the literature on the effects of daylight hours on reproduction in animals, has put forward as the most likely mechanism of pituitary stimulation that, with the eye region as receptor, the impulses are transmitted through a neural pathway and by humoral means as well. Harris (1947) has suggested that excitation of the anterior pituitary gland is effected by nerve stimulus to the hypothalamus and then by humoral stimulus to the pituitary. This suggests that the stimulating factor may be the change-over from long-dark to short-light, or short-dark to long-light in much the same way that nerve muscle is stimulated at the make and break of the current. For example, in the case of electrical stimulus of nerves affecting ejaculation, Gunn (1936) found a sequence of about 5 sec. on and 10 sec. off to be very much more effective than other sequences.

Light and dark may act as two contrasting factors for recording long and short (a sort of dot and dash, or make and break system) on the nerves in the region of the eye and thence to the pituitary of the animal and so affect the output of gonadtropic hormones. For example, in group IV (11-hr. light–13 hr. dark) the contrast is short-light: long-dark and results in anoestrus; by giving artificial light for one hour (as in group I) so as to divide the long period of darkness into two short periods, a long-light: short-dark contrast is produced, which accelerates the onset of oestrus without greatly altering the total quantity of light or darkness in the 24 hr.

In accelerating the onset of oestrus in ferrets there seems little doubt that:

(1) The total daily quantity of light is not the controlling factor. On the other hand, the sequence of light and darkness is extremely important.

(2) A gradually increasing plane of light or decreasing plane of darkness is not essential in itself, but is merely the means whereby the change-over from a short-light: long-dark sequence to a long-light: short-dark is effected in nature.

(3) Long-light and short-dark are purely relative terms as far as the animal is concerned, and are defined from the animal's reception point of view by contrast with each other, and not in terms of total hours.

1948–9 *experiments.* A just and valid criticism of the 1947–8 experiments is that the number of animals used in each group was small. It is only fair to point out that this was not due to bad design of the experiment, but followed from the circumstance that the decision to embark upon these experiments was taken very late in the year. Then, because it was necessary to commence light treatment as long as possible before the shortest day (21 December) after which natural daylight would be changing over towards the long-light: short-dark sequence, there was not sufficient time available to obtain a supply of suitable ferrets. Hence it was decided to commence the experiment with those animals already available in order to avoid losing a year.

Results obtained on sheep (Hart, 1950) during the summer of 1948, however, confirmed the conclusions obtained with ferrets in the winter of 1947–8. By using larger numbers of ferrets in the 1948–9 experiments, it was hoped to confirm the results obtained in 1947–8. In addition, it was planned to obtain information about the effects of giving the animals several stimuli during one 24 hr. period compared with nature's single light–dark stimulus per 24 hr., for it seemed possible that by putting in several stimuli per 24 hr., the response might be somewhat similar to the tetanus contraction of muscle and so produce a quicker effect.

Another problem to be investigated, was whether anoestrus could be induced in oestrous ferrets by reversing these light–dark sequences and, if so, what would be the latent period. A comparison of the lengths of the latent periods between the change in sequence and the onset of oestrus (or of anoestrus) might give some indication of the slope of the 'depth of anoestrus curve' for these animals (Hart, 1950).

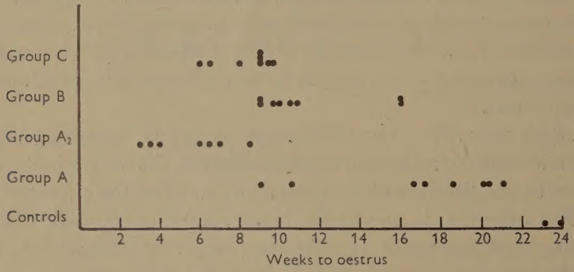

Fig. 8. Scatter plot showing latent periods to oestrus of all animals.

The effect on the breeding season of animals kept under continuous light was another matter which required to be studied; unfortunately the converse to this, namely that of continuous darkness, presented too many practical difficulties of management, e.g. of cleaning and feeding, to allow it to be carried out. It was thought that continuous hooding (Bissonnette, 1935) would not be a practical proposition.

None of the ferrets used in these experiments had experienced an oestrus cycle previously as they were only born in June 1948. Since they were entering the normal anoestrous period of the year at the commencement of treatment, they were considered to be very deep in anoestrus, and at one of the most unresponsive stages of their life from the breeding season point of view.

Scatter charts showing the results obtained on all the animals used are given in Figs. 8 and 9. From these it will be seen that once again 100% oestrus was achieved in groups B, C and A_2, where constant light rhythms were used. This again shows that increasing or decreasing planes of light or darkness are not necessary, and confirms the previous year's findings as detailed above. In group A, however, the results must be regarded as rather inconclusive in that the purpose of the continuous light treatment was defeated by allowing natural daylight to be added to the continuous artificial light over the first 11 weeks of this experiment. Nevertheless carefu analysis of this failure raises some interesting possibilities which will be discussed later. In groups A_1 and C_1, where animals in full oestrus were placed under constant light-dark sequences aimed at inducing anoestrus, again all the animals responded without exception.

Table 1

Group	Light-dark sequence	Mean latent period of group 'weeks'
Mean latent period from beginning of treatment to oestrus		
A	Natural daylight + continuous artificial light	16·6
B	8 hr. light–4 hr. dark	11·25
C	16 hr. light–8 hr. dark	7·67
A_2	4 hr. light–2 hr. dark	5·5
Controls	Natural daylight	23·5
Mean latent period from beginning of treatment to anoestrus		
A_1	4 hr. light–20 hr. dark	3·91
C_1	8 hr. light–16 hr. dark	6·3

Examining the latent periods from the beginning of the light treatment to the onset of oestrus of all groups, as shown in Table 1, it is obvious that in groups B and C there has been a significant acceleration in the onset of oestrus by more than 12 weeks as compared with the controls. This margin would have been larger if all the controls had been included for comparison; only the first two of these which came into oestrus were recorded since the differences were so obvious and extreme, and the animals were required for another experiment.

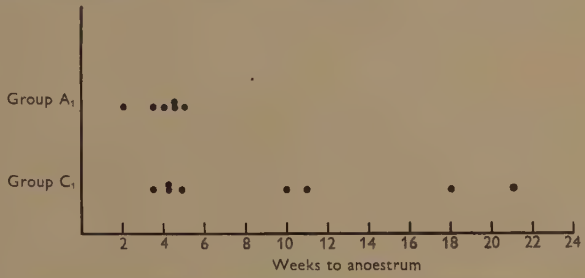

Fig. 9. Scatter plot showing periods to anoestrum. All animals used are plotted.

The mean latent period of 5·5 weeks in group A_2 cannot be related to any control group. Controls were obviously not available at the time of the year (30 July) when the light treatment for this group commenced, for the normal summer oestrous period for ferrets under natural conditions occurs at this time of year. Nevertheless, it is felt that there is no reason to doubt that group A_2 would have shown significant acceleration over controls if it had been possible to run this group at the same time as groups A, B and C.

In comparing the mean latent periods between groups, group A (continuous light) has to be excluded for reasons previously stated and also group A_2 (4 light–2 dark) because of the possibility of residual effects. This leaves groups B (8 light–4 dark) and C (16 light–8 dark), where it would appear that there is an acceleration of C over B to quite an appreciable extent, the amount being 4 weeks if all animals in group B are included, and 2 weeks if the last two ferrets of this group (which were 16 weeks before responding) are excluded as not being consistent. On this evidence it would appear that there is no 'tetanus effect' induced by the giving of more than one stimulus in each 24 hr. period, for the onset of oestrus has not been speeded up. It may well be, however, that with only two stimuli per 24 hr. given to group B, there was not sufficient step-up in the number of stimuli, and something of the order of 16 or 8 stimuli per 24 hr., which could be obtained by making light–dark sequences of 1:½ or 2:1 hr. would prove more efficient in obtaining a 'tetanus effect'. The response of group A_2 has shown that the ratio 4 light:2 dark in a 6 hr. period is sufficient to activate the pituitary mechanism controlling the onset of oestrus, but whether a further reduction in period will still be sufficient to provide this stimulus has yet to be shown. If we accept the hypothesis that the pituitary is contrast sensitive to long and short, expressed in terms of light and darkness, and not to total quantity of light, then such reduction might well be possible. It has also been demonstrated beyond doubt that a ratio of 2 parts or more of light to 1 part darkness is sufficient to provide the long-short rhythm, but just how far this ratio may be narrowed towards equality has yet to be ascertained. There is no doubt, however, that this ratio may be narrowed as close as 10 parts light to 6 parts darkness and still be effective, this being shown by the results of group I in the 1947–8 experiments.

In considering the latent periods from beginning of treatment to the onset of anoestrus shown in Table 1, it has to be remembered that no controls could be used. The two treatments were given, first, to prove that the pituitary could be influenced to induce anoestrus in fully oestrous animals by reversing the light–dark ratios employed for producing oestrus. Secondly, once this had been shown to be effective it then became possible to carry out the experiment with the A_2 group (on a 4 hr. light–2 hr. dark sequence), keeping the remainder of the animals on the group C_1 sequence (of 8 light–16 dark) as a type of negative control against A_2. A study of the latent periods in these two groups (A_1 and C_1, Table 1) gives some information on the possible slope of the sexual season curve towards anoestrus, for even including the last two ferrets to respond in group C_1 although obviously inconsistent, the mean latent periods of groups A_1 and C_1 are substantially shorter than those of groups B, C and A_2. This seems to indicate that the slope of the sexual season curve (Hart, 1950) is much steeper in proceeding from oestrus to anoestrus than from anoestrus to oestrus in the ferret. The differences between groups A_1 and C_1 do not appear to be sufficient to warrant a definite conclusion being drawn at this stage of investigation.

As has already been pointed out, group A (continuous light) cannot be regarded as having demonstrated what it was originally intended to show, namely that a sequence of light–dark was necessary to induce oestrus by stimulation of the anterior pituitary. These animals took longer to come into oestrus than those which had an artificial light–dark sequence of 2:1 (see Table 1), but they showed a shorter latent period than the controls exposed to natural daylight. Unlike the other groups, group A was exposed to natural daylight (which at that time of year was increasing) in addition to the continuous artificial light. It was then realised that the natural daylight might be having an influence on the results and so a series of careful readings of light intensities were made at different times of day in the pen. The results of these readings are shown in Table 2 and it can be seen that even on dull days the total light intensity was twice that of the artificial light alone, whilst on an ordinary bright day the total light intensity was increased as much as three times. These figures showed that the animals in group A were in effect receiving some type of contrast stimulus of long and short owing to the light being of two intensities. That is, the relatively weak light of 29 f.c. from the artificial light alone was being contrasted with the relatively strong light of 60 f.c. and above due to artificial light plus daylight. It is considered that this slight contrast effect every 24 hr. period was sufficient eventually to bring about the onset of oestrus, but with a far longer latent period than in the other experimental groups, where the contrast between light and darkness was total, complete and perfectly controlled.

Table 2. *Sample of light intensity readings* (*group A*)

Date	Time (p.m.)	Light intensity in pen (foot-candles)	Light intensity outdoors (foot-candles)	Remarks
1 March	1	95	250+	Bright day
1 March	7	29	—	Completely dark
2 March	1	100	250+	Bright sun
2 March	5	31	135	Dusk
2 March	7	29	—	Completely dark
3 March	1	60	200	Dull day
3 March	5	30	125	Overcast and dusk
3 March	7	29	—	Completely dark

Incidentally it should be recorded that blindness gradually began to affect some of the animals after about four months of constant artificial light treatment, and this led to wasting and in a few cases the eventual death of some of the affected animals. There were several cases where only one eye was affected so it may be possible that the blindness was not wholly due to the light but to some organism.

SUMMARY

Evidence accumulated during two years' work and experience in photoperiodicity in ferrets suggests the following conclusions:

1. Oestrus or anoestrus may be induced by influencing the activity of the pituitary gland; this is effected through a contrast-sensitive mechanism which is stimulated by light:dark sequences. The total daily quantity of light is not the controlling factor.

2. In order to obtain the long-light:short-dark ratio necessary for an oestrus-activating sequence, the ratio should be in the proportion of two parts or more of light to one part darkness and reversed if it is desired to induce a state of anoestrus.

3. Slowly increasing or decreasing planes of light are not an essential part of an oestrus-activating sequence.

4. Because ferrets can be brought into anoestrus quicker than they can be brought into oestrus it is considered that the sexual season curve is more acute when entering anoestrus than when entering oestrus.

5. Doubling the number of light–dark contrast stimuli from one every 24-hour period to two every 24-hour period produced no acceleration of the onset of oestrus nor any indication of a 'tetanus effect'.

My thanks are due to the Agricultural Research Council for financial assistance, to Dr John Hammond for his help and encouragement, and to the staff of the School of Agriculture, Cambridge, for their assistance in attending to the animals.

REFERENCES

BISSONNETTE, T. H. (1932). *Proc. Roy. Soc.* B, **110**, 322.
BISSONNETTE, T. H. (1935). *J. Exp. Biol.* **12**, 315.
BORTHWICK, H. A. (1947). *U.S. Yearb. Agric.* 1943–7, p. 273.
GUNN, R. M. C. (1936). *Bull. Coun. Sci. Industr. Res.*, Aust., no. 94.
HAMMOND, J. Jr. (1949). Personal Communication.
HARRIS, G. W. (1947). *J. Endocrinol.* (*Proc.*), **5** (3), 17.
HART, D. S. (1950). *J. Agric. Sci.* (in the press).
HILL, M. & PARKES, A. S. (1933). *Proc. Roy. Soc.* B, **113**, 537.
MARSHALL, F. H. A. (1940). *J. Exp. Biol.* **17**, 139.
YEATES, N. T. M. (1949). *J. Agric. Sci.* **39**, 1.

A BIFUNCTIONAL SINGLE MOTOR AXON SYSTEM OF A CRUSTACEAN MUSCLE

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(With Four Text-figures)

INTRODUCTION

The muscles of decapod crustaceans have been shown to possess a number of special features. Among these is the remarkable property which enables many of them to contract in two distinct ways, by fast and by slow contractions. Several explanations have been offered to account for this property (Lucas, 1917; Blaschko, Cattell & Kahn, 1931; Wiersma, 1933; Pantin, 1936*a*, *b*; Katz, 1936; Wiersma & Van Harreveld, 1938*b*; Katz & Kuffler, 1946).

It was shown that in all muscles which have both a slow and fast contraction, and which have been investigated by the method of isolating and stimulating single axons, it is possible to obtain one or other contraction type by the stimulation of one or other of the two motor fibres which form the sole motor innervation for most of the leg muscles. In the case of the main flexor muscle of the carpopodite which receives, instead of two, four motor fibres, each of these four was found to elicit a contraction with specific properties.

Hence it became likely that each contraction type of a given muscle was caused by a special motor fibre.

Openers (abductor of the dactylopodite) and stretchers (extensor of the propodite) were found to receive a single motor fibre in every case in which single-fibre analysis was made. This would then indicate that these muscles possessed only a single type of contraction. Most of the results obtained supported this view, as there was in most species no noticeable difference between the opener contractions and the slow contractions of the doubly motor-innervated muscles of the same species. However, in older experiments with the hermit crab (Wiersma & van Harreveld, 1934*a*, *b*, 1935), it was shown that two types of contractions were performed by its opener. Since this species had never been analysed for motor fibres, it was possible that its opener did in fact receive two motor fibres.

This assumption remained unquestioned until recent evidence (Wiersma & Adams, 1950) indicated that the general rule need not apply to openers. The present investigation was undertaken to determine the number of motor fibres innervating the opener of *Eupagurus bernhardus* L., and to obtain further information concerning the two contraction types shown by this muscle.

METHODS

Single motor fibres of the leg muscles of *E. bernhardus* L. were prepared according to the usual technique (Wiersma, 1941), and stimulated through micromanipulated platinum electrodes. In these experiments both claws and the first two walking legs have been used. For mechanograms of the opener the large claw was most satisfactory, since it showed the desired phenomena more clearly than the other legs. The differences were of a quantitative nature only.

Low-frequency stimulation was obtained with a variable interrupter in the primary circuit of an induction coil. Extra shocks were intercalated by a second coil, the secondary of which was connected to the same stimulating electrodes through which the low-frequency-background-stimulation (3–20 per sec.) was given. A modified Lucas contact breaker with four contacts was used in a number of experiments, but in many of these only one or two of its contacts were connected. No exact time calibration was available for this instrument.

Contractions were registered isotonically, the lever being fastened to the dactylopodite. Closer muscles have been used as a control in some cases. All claws were obtained from freshly caught animals in excellent condition during the summer of 1949 at the Marine Station in Den Helder.

RESULTS

A. *Staining with methylene blue*

Opener muscles (as well as other muscles) of the leg were stained with methylene blue in order to determine the number of nerve fibres innervating them. Staining was often satisfactory, though never so good as in the coconut crab, *Birgus latro* L. (Wiersma, 1949).

Three fibres were regularly observed in the closer, the bender and the extensor, as found in *Birgus*. Both in the opener and in the stretcher only two nerve fibres stained with certainty. Many preparations of the opener have been made and stained for different lengths of time with different concentrations of dye. In examining them, high-power magnification was sometimes used in order to try to find a third fibre as is present in *Birgus*. In *Birgus* this third fibre is small and acts as an inhibitor; it could be prepared functionally in the ischiopodite, but not in the meropodite because it branches there. The conclusion that in *Eupagurus* the opener does not receive a branch from this fibre, though supported both by anatomical and physiological evidence (see below), nevertheless cannot be regarded as certain. Thin fibres are sometimes very difficult to stain, and the course of a fibre may preclude its functional isolation in the meropodite. Yet it is not unlikely that such differences exist in innervation schemes among Anomura (Wiersma, 1949).

B. *Determination of the number of fibres innervating the opener and other muscles*

It was of primary importance for this investigation to decide whether or not one motor fibre formed the sole innervation of the opener muscle. In Anomura as in

Astacura, there occur in the meropodite two clearly separated bundles of nerve fibres, one thick and the other thin. Stimulation of the latter causes opening. In previous investigations (Wiersma & Van Harreveld, 1934*a*) on *Eupagurus*, the thick bundle had been cut. The opener contractions obtained were thus due to the stimulation of the whole thin bundle. The results might have been influenced either by the presence of two (or more) motor fibres for the opener in this bundle, or possibly by the presence of an inhibitor for the opener. Axon isolation in this bundle was therefore carried out with special care. The results were absolutely uniform; only one fibre could be found in this bundle which had any effect on the opener muscle. This fibre, which has been isolated many times, always caused a simultaneous contraction of opener and stretcher. The only other efferent fibre found in this bundle is the stretcher inhibitor, which runs side by side with the opener-stretcher motor axon. The stimulation of this fibre has no influence at all on the opener. It must therefore be concluded that stimulation of the thin bundle as a whole will have exactly the same effect on the opener as stimulation of the isolated motor axon (see also below).

A search has also been made for the efferent axons in the thick bundle. As expected, two motor axons for the closer and two for the bender were found. Stimulation showed that each of these muscles is innervated by one fast and one slow motor fibre. In addition, a fibre was prepared whose sole function was inhibition of the opener muscle, and a further fibre which, on simultaneous stimulation of the appropriate motor axons, could be shown to inhibit both closer and bender contractions. But stimulation of the latter fibre did not influence either opener or stretcher contractions. It was also found that stimulation of the isolated stretcher inhibitor from the thin bundle was without result on either closer or bender contractions.

C. *Stimulation of the isolated opener-stretcher motor axon with long-lasting direct current*

Since only one motor axon is present in the thin bundle, stimulation of the isolated single fibre should give the same effect as similar stimulation of the whole bundle. As found in previous experiments (Wiersma & Van Harreveld, 1934*a*), stimulation of the whole bundle did not give the smooth tetanus that is usually obtained when only one motor fibre is stimulated with a long-lasting direct current. Rather, it gave a veratrine-like contraction, consisting of a fast rise and a slight fall, followed by a slow rise before relaxing. Such contractions occur in doubly motor-innervated muscles only when both motor fibres are stimulated together. Then the first quick rise is due to the fast fibre, which usually fires for a very short time, so that relaxation occurs before the shortening caused by the continued firing of the slow fibre.

But long-lasting direct-current stimulation of the totally isolated motor axon of the opener of *Eupagurus* did in fact give rise to a veratrine-like contraction (Fig. 1). The large claw showed this phenomenon more clearly than did the other legs, because the relaxation between the fast and the slow phases of the contraction was more pronounced. On repetition of stimulation the relaxation became less (Fig. 1), and eventually the slow part of the contraction followed the fast without any intermediate relaxation.

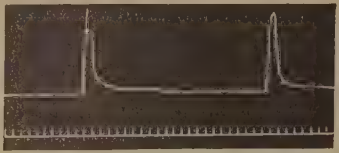

Fig. 1. Opener, prepared axon. Stimulation with constant current of 1 sec. duration, near threshold of twitch contraction. Veratrine-like shape, isotonic. On repetition the slow part becomes relatively more pronounced. Time in seconds.

D. *The effect of intercalated shocks*

Since stimulation of the whole thin nerve bundle gives contractions which are identical with those obtained after the isolation of the motor fibre, some of the experiments under D and E were carried out by stimulating the undivided thin bundle. This was done in order to prevent any fatigue of the opener muscle. Frequent checks with isolated fibre preparations showed that for this type of experiment also the results were the same in the two cases.

In a previous paper (Wiersma & Adams, 1950) it was shown that when a slow fibre is stimulated at a constant frequency and a single extra shock is given, there is never any marked effect of such intercalation and often no sign of it can be detected. When this same type of experiment is performed with a fast fibre, however, the intercalated shock often causes a very noticeable twitch. These extra twitches are, in some cases, immediately followed by a relaxation to the tetanus level; or, in others, followed by a complete or partial maintenance of the newly reached level of shortening. In openers of different species the results were different: in the crab, *Pachygrapsus*, intercalated shocks had practically no effect on the mechanogram, whereas in the crayfish, *Cambarus*, there was a distinct effect in many cases.

If in *Eupagurus* the first phase of the veratrine-like contraction is comparable to a fast contraction, intercalated shocks should have a noticeable effect. This proved, indeed, to be the case (Fig. 2) . The amount of shortening achieved by a given intercalated shock will, of course, depend on its time relations with the shocks preceding or following it, and this factor is not controlled. Thus some of such shocks will have no effect, because they fall within the refractory period of a previous one, or blot out a following shock by their own refractory period. Also, when a nerve impulse does result, there will be an optimum interval, at which the largest 'trigger' effect occurs. It was found that a noticeable relaxation after the twitch-like contraction was obtained only when the muscle shortened considerably in response to the intercalated shock; smaller contractions were sustained (Fig. 3). In no case was a twitch followed by a relaxation to the 'base' line, as was the case in several fast systems.

Different frequencies have been used for the background stimulation, and trigger effects have been found with every frequency that gave a visible contraction. It is important to note that very small, but complete or almost complete, tetanus was obtained with a frequency as low as 2 per sec. (Fig. 2), which is a feature of slow systems.

Some experiments have been performed with the isolated slow and fast closer axons. As expected, intercalated shocks were without effect in slow contractions and in the fast contractions gave noticeable trigger responses, of a magnitude comparable with those in the opener.

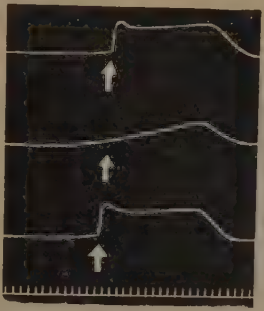

Fig. 2. Opener, isolated axon. Stimulation frequency 3 per sec. for 20 sec. At the arrows, 10 sec. after start of faradic stimulation, a single opening induction shock is given, which results, in first and third trials, in a veratrine-like shaped increase of contraction, but is without effect in the second trial.

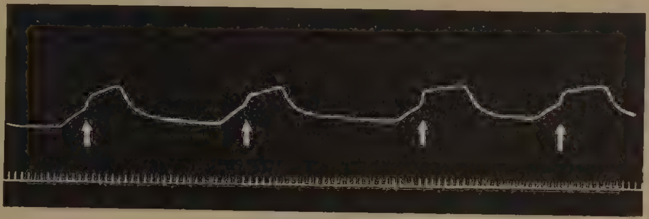

Fig. 3. Opener. Stimulation of thin bundle, with a frequency of about 10 per sec. At the arrows a single intercalated opening shock is given. Notice differences in effect in the different contractions, the third trial giving a slightly veratrine-like shape, whereas the others are followed by a sustained contraction of different magnitudes.

E. *Effect of spacing of stimuli*

In the paper quoted (Wiersma & Adams, 1950) it was shown that in cases where intercalated shocks had a large effect, the spacing of stimuli in time is of very great importance. This means that if, for instance, 20 stimuli are given per second the result will be different according to whether the intervals are uniformly $\frac{1}{20}$ sec. or whether the intervals are, say, alternately, $\frac{1}{80}$ and $\frac{7}{80}$ sec. The contraction with the latter distribution will be considerably larger than that with the former. In slow systems, on the other hand, such changes in spacing do not give rise to any significant differences in the contraction.

In the present investigation the total number of spaced stimuli had to be restricted to four, which proved only just enough. Each contact of the contact breaker was made to break the primary of an induction coil. The four secondaries of these coils were connected to one pair of stimulating electrodes. The first and last contacts were given fixed positions; the interval may have been of the order of some 20 msec. The other two contacts were either spaced so that all intervals were equal, or so that the second stimulus came immediately after the first and the third immediately before the fourth. It was found that under the conditions of these experiments all four contacts were necessary to obtain a contraction; with three no visible response was obtained (Fig. 4; 4 and 5). The contractions with equal spacing were always smaller (Fig. 4; 2, 3, 6, 7), often about half the size of those obtained by crowding the second near the first and the third near the fourth (Fig. 4; 1 and 8). This fact shows that spacing has in this muscle a very pronounced effect, as would be expected if a fast system were present.

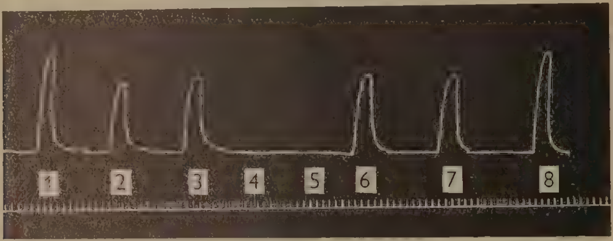

Fig. 4. Opener, single axon. Effect of spacing of shocks. 1, result of four shocks, spaced about as follows: interval 1st–2nd and 3rd–4th 2 msec., interval 2nd and 3rd 18 msec. 2, 3, 6 and 7, result of even spacing of four shocks, intervals 5 msec. 8, same spacing as in 1. Notice increased size of 1 and 8 as compared to other contractions. In 4, the first, in 5 the last of the shocks (equal spacing) were left out. No visible contraction on three impulses. Time ⅕ sec.

DISCUSSION

From the foregoing results, it follows that the opener receives only one motor fibre and that this one fibre can elicit, depending on the way in which it is stimulated, two types of contraction, which resemble the fast and slow contractions of other muscles. This finding leads to a reconsideration of the problem of fast and slow contractions and necessitates a more extensive discussion.

The most obvious difference between fast and slow contractions is found in the closer muscle of the crayfish, where it was first described. Here, a single impulse in the fast fibre will cause a twitch contraction, whereas several impulses are needed before stimulation of the slow fibre will cause a visible smooth tetanus. However, this is a specialized case. In most doubly motor-innervated muscles, a number of impulses are needed in the fast fibre before a twitch is produced. In several cases this number is only two, but since in certain crabs (*Cancer*) a good twitch is obtained only with a larger number, the two systems are no longer easily distinguishable. At low frequencies, the fast may even produce smaller contractions than the slow in these cases (Wiersma & van Harreveld, 1938*b*).

Another difference, which has been found so far to be reliable, is that intercalated shocks will cause a pronounced 'trigger response' in fast systems, but not in slow. Related to this is the fact that the spacing of impulses has a great influence in fast systems but not in slow; those fast systems which respond on a single impulse are, however, exceptions.

The muscle action potentials are also indicators of the type of contraction, for in slow systems they are smaller than in fast. But in both systems they show electrical summation in the way that contractions can show mechanical summation. This summation is considered to be related to the fact that these action potentials are local phenomena.

If we now consider, in the light of the above differences, the results obtained on the opener system of *Eupagurus* and described in this and previous papers, the conclusion seems inevitable that the contractions of this muscle are of a type which includes properties of both fast and slow neuro-muscular systems. It shares with slow systems the property that on low-frequency stimulation a gradual shortening develops. This shortening is perhaps somewhat more in the nature of an incomplete tetanus than in other slow systems, but it lacks the sudden increases and decreases in strength which are characteristic of fast systems when stimulated near the lowest effective frequency. However, as has been shown, it shares with fast systems the sudden increases in contraction produced by intercalated stimuli and the great sensitivity to pattern.

Again, in the muscle action potentials, it was previously found that at a relatively low frequency of stimulation there is a gradual growth from zero, as in slow systems, and not a growth from a considerable initial potential as in fast systems. In the case of the opener muscle, however, this growth may be rather more pronounced than in slow systems. Then again, a sudden burst of impulses will result in a big action potential 'spike', which looks exactly like that of such fast systems as are found in *Randallia*, and not like any known 'slow' potential.

The ability of a single motor axon to cause contractions with both slow and fast properties may be explained in several ways. According to Katz & Kuffler (1946), fast contractions are at least sometimes accompanied by conducted action potentials. If one accepted that the fast phase of the contraction was due solely to such conducted potentials in this muscle, the double contraction type would be readily explained. To the present author, there are great difficulties in the way of this hypothesis. Conducted action potentials would of necessity give rise to diphasic action potentials under most leading off conditions. From the previous results (Wiersma & Van Harreveld, 1935, fig. 3), it is obvious that the first part of the action potential configuration which accompanies a veratrine-like contraction, is just as monophasic as the later part. Monophasic action potentials have been found in other muscles as well as in the opener of *Eupagurus*, and the present author agrees with Wright (1949), that conducted action potentials have not been proved to exist in any crustacean system. Roeder & Weiant (1950) have also come to the conclusion that the action potential accompanying fast contractions in insect muscles are not conducted, though the time relations of these contractions are certainly shorter than those of even the fastest twitches in crustacean muscles.

A second possibility would be that two types of contraction are due to two kinds of muscle fibres, each with its own kind of facilitation. This possibility is not excluded by direct evidence. However, it seems unlikely, because of the small number of muscle fibres in the opener and the fact that in doubly motor-innervated muscles the same muscle fibres are responsible for both contractions (Van Harreveld, 1939). The mechanisms by which the two fibre types are brought into contraction would still have to be the same as in the hypothesis which will be favoured here.

The third possibility is that there are two types of nerve endings on the same muscle fibre. The first type becomes activated when impulses at a low frequency reach it, since these endings have small but long-lasting facilitation. The second type becomes activated only when a burst of impulses at high frequency reaches it, because it has strong but short-lasting facilitation. In other words, the slow and fast endings as present in doubly motor-innervated muscles would here have a common axon instead of a fibre for each type. The postulation of two types of ending, instead of one which would combine the properties of a fast and slow ending, is based upon the veratrine-like contractions and the effects of intercalated shocks. If the same endings which are responsible for the slow phase of the contraction were also responsible for the twitch phase, the facilitation of the former by a twitch would be so large that no relaxation could be expected to precede the subsequent increase in tension. Also, the action potentials accompanying a veratrine-like contraction are clearly divided into two parts. After the first summated action potential, there is a sudden drop followed by a renewed action potential facilitation (see Wiersma & Van Harreveld, 1935, fig. 3). If only one process were present, one would expect a gradual diminution of the action potential, as the original high amount of facilitation disappears.

The two types of endings will, of course, be excited at the same time, but as stated, with low frequencies, contractions will take place only at the 'slow endings'. With higher frequencies, the 'fast endings' will also contribute to the contraction. On longer lasting stimulation it becomes impossible to decide what part each type of ending plays; but when only a few impulses follow each other quickly, the contribution of the slow endings will be negligible, and thus twitches will be the product of the fast endings almost exclusively.

It might seem that such a system of fast and slow, depending upon a single nerve fibre, would have advantages over one in which two nerve fibres are necessary. However, since systems of this type can only function when a relatively large number of impulses is given, sufficient to trigger the fast contraction, the delay in the onset of contraction is necessarily increased. Further, separation of the two systems may result in a much greater overall economy, especially of contractions due to an intermediate frequency. This may explain why the 'bifunctional single axon system' has developed only in muscles of relatively small size, such as the openers, which have a rather insignificant metabolism. These muscles are used mostly in preparatory movements. Their antagonists, such as the closers, in which considerable power is developed, are doubly innervated.

SUMMARY

It is shown that the opener muscle of the hermit crab, *Eupagurus bernhardus* L., receives a single motor axon. Stimulation of this axon results, when appropriate stimuli are used, in two types of contractions comparable with the fast and slow contractions of doubly motor-innervated crustacean muscles. The theoretical implications of this finding are discussed and a hypothesis offered to explain the mechanism which makes the two contraction types possible. The physiological and anatomical features of the innervation of the four most distal muscles in the legs of *Eupagurus* are described.

REFERENCES

BLASCHKO, H., CATTELL, M. & KAHN, J. L. (1931). *J. Physiol.* **73**, 25.
HARREVELD, A. VAN (1939). *J. Exp. Biol.* **16**, 398.
KATZ, B. (1936). *J. Physiol.* **87**, 199.
KATZ, B. & KUFFLER, S. W. (1946). *Proc. Roy. Soc.* B, **133**, 374.
LUCAS, K. (1917). *J. Physiol.* **51**, 1.
PANTIN, C. F. A. (1936*a*). *J. Exp. Biol.* **13**, 111.
PANTIN, C. F. A. (1936*b*). *J. Exp. Biol.* **13**, 148.
ROEDER, K. D. & WEIANT, E. A. (1950). *Fed. Proc.* **9**, 108.
WIERSMA, C. A. G. (1933). *Z. vergl. Physiol.* **19**, 349.
WIERSMA, C. A. G. (1941). *J. Comp. Neurol.* **74**, 63.
WIERSMA, C. A. G. (1949). *Physiol. Comp. Oecol.* **1**, 68.
WIERSMA, C. A. G. & ADAMS, R. T. (1950). *Physiol. Comp. Oecol.* **2**, 20.
WIERSMA, C. A. G. & HARREVELD, A. VAN. (1934*a*). *Arch. néerl. Physiol.* **19**, 426.
WIERSMA, C. A. G. & HARREVELD, A. VAN. (1934*b*). *Arch. néerl. Physiol.* **19**, 458.
WIERSMA. C. A. G. & HARREVELD, A. VAN. (1935). *Arch. néerl. Physiol.* **20**, 89.
WIERSMA, C. A. G. & HARREVELD, A. VAN. (1938*a*). *J. Exp. Biol.* **15**, 18.
WIERSMA, C. A. G. & HARREVELD, A. VAN. (1938*b*). *Physiol. Zoöl.* **11**, 75.
WRIGHT, E. B. (1949). *J. Cell. Comp. Physiol.* **33**, 301.

GIANT AXONS AND SYNERGIC CONTRACTIONS IN *BRANCHIOMMA VESICULOSUM*

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From the Marine Biological Laboratory, Plymouth

(*Received 2 June* 1950)

(With Eight Text-figures)

INTRODUCTION

Conduction in the giant axons of annelids has been clarified by the work of Stough, Eccles and colleagues, Bullock, Rushton and others (reviewed by Nicol, 1948*a*). They are now known to be motor axons, or at least nerve fibres on the efferent side of reflex arcs, and they intermediate quick withdrawal movements effected by synergic contractions of widespread longitudinal muscles. Probably the most highly developed axons of this kind occur in the Serpulimorpha (Nicol, 1948*a*, *b*, *c*, *d*). Our knowledge of the physiological activity of these nerve fibres, as components of a neuro-muscular unit, is still incomplete, however, and the sabellid worm *Branchiomma vesiculosum* (Montagu) has been investigated, as a suitable experimental animal. The present paper gives an analysis of giant axon functioning in the intact animal as revealed through muscular activity. A few observations on the functioning of the giant fibres in *Myxicola infundibulum* (a related species) have already been published (Nicol, 1948*c*), and will be referred to in the present account. Budington (1902) described the results of stimulating pieces of the earthworm which contained nerve cord. His records show contractions of the longitudinal muscles, and it is probable that he was stimulating the giant axons of the animal.

An account of the histological organization of the giant axons of *Branchiomma* is the subject of a complementary paper. This is summarized as follows. There are two giant axons in this species (Fig. 1), and each axon arises from a large cell in the posterior part of the supraoesophageal ganglia. The two fibres decussate in these ganglia, and each then descends through the circumoesophageal connective to reach the ventral nerve cord. A giant axon lies in each half of the nerve cord and runs to the posterior end of the abdomen. The giant axons are certainly syncytial structures connected with a number of nerve cells, but no attempt has been made to discover all their neurocytons.

An important feature of the organization of the giant axons of *Branchiomma* lies in the arrangement and distribution of the motor branches to the muscles. In each segment a giant axon gives off lateral branches which proceed directly to the ipsilateral longitudinal muscles of that segment (Fig. 1). These motor branches run on the internal faces of the muscle masses, and give off finer twigs which descend into the muscle mass among the muscle fibres. The giant axons in the supraoesophageal ganglia are about $10\,\mu$ in diameter; in the thoracic nerve cord they increase to $230\,\mu$, and gradually taper off posteriorly, to reach fine dimensions of about 7 μ or less in the tail. The motor branches are about 15 μ in diameter at their origins in the nerve cord, and they dwindle to fine filaments among the muscles.

The structure of polychaete muscles has been reviewed by Prenant (1929). Apparently the longitudinal muscles of *Branchiomma* and other sabellids are nematoid in form and consist of longitudinal ribbons which are attached to connective tissue membranes, and are invested by connective tissue fibres. Each ribbon contains a flattened contractile plate, folded on itself to form a compressed U. The plate is the contractile element and contains myofibrils, but lacks striations. Sarcoplasm bulges from an orifice in the plate, surrounds the fibre as a sheath, and contains a nucleus; along one edge of the fibre the sarcoplasm may form a peculiar frilled membrane. Circular and parapodial muscles appear to be similar. Consequently, these muscles seem to be rather unusual types of smooth muscle (Brunotte, 1888; Hanson, 1948; Jourdan, 1887; Prenant, 1929). Both Brunotte and Prenant regarded the longitudinal muscles as syncytial structures in which the contractile plates lie in an anastomosing sarcoplasm.

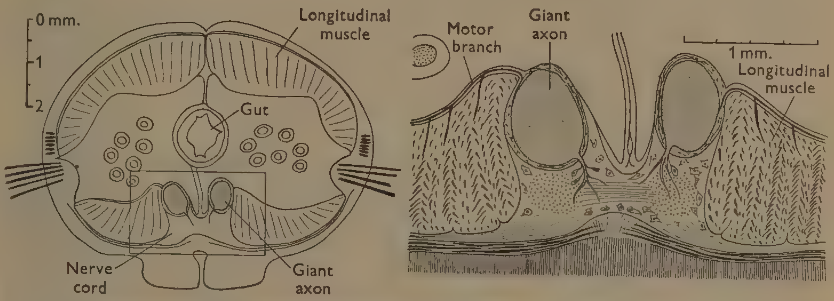

Fig. 1. Semi-diagrammatic drawings of sections of *Branchiomma vesiculosum* to show the arrangement of the giant axons and longitudinal muscles. Left, transverse section of the body in the anterior abdominal region. The rectangle in the lower centre is shown enlarged on the right.

METHODS

Specimens of *B. vesiculosum* used in this investigation were kept under circulation in the laboratory. For an investigation of the functioning of the giant fibres and longitudinal muscles the entire animal forms a convenient neuro-muscular preparation. Animals were removed from their tubes and studied in sea water. When the animal is stimulated mechanically by touching it with a glass rod, it shortens abruptly. This reaction involves a complete reflex arc beginning with epidermal receptors which fire the giant axons in the nerve cord, the giant axons forming the efferent side of the arc. Electrical stimuli delivered to the surface of the animal stimulate the giant axons directly, resulting in synergic contraction of the longitudinal muscles and shortening of the animal.

For recording contractions both isotonic and isometric levers were used. A hook was passed transversely through the anterior end of the animal and connected to the recording lever. The anterior end (head and thorax) is rather firm and hard, owing to dense musculature and a turgid endoskeleton supporting the branchial crown. The posterior end was pinned down on a cork surface. Isotonic levers were lightly weighted to return the animal to its original length and to cause the writing point to return to base-line. Certain characteristics of the preparation that present difficulties in recording will be referred to in the observations. Here it is noted that the animal is very extensible. Under stimulation and recording it gradually fatigues and elongates, and it is necessary to keep adjusting the isotonic lever to maintain some light tension and to take up the slack. Again the tissues of the animal, particularly those of the posterior abdomen, are soft and easily torn; this restricts considerably the tension to which the animal can be subjected in making a taut line to an isometric lever. Despite these limitations favourable preparations give clear-cut results.

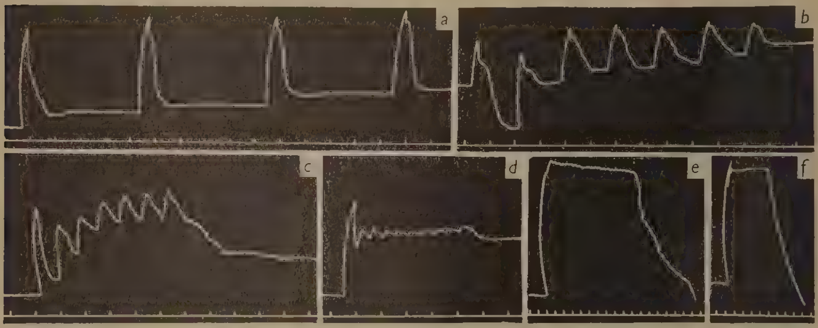

Fig. 2. Isotonic contractions following stimulation of the giant axons of *Branchiomma vesiculosum*. Upper line, contractions of the animal; lower line, time scale at 1 per sec. Stimulation frequencies: *a*, 12 per min.; *b*, 36 per min.; *c*, 62 per min.; *d*, 124 per min.; *e*, 164 per min.; *f*, 13 per sec.

Electrical stimuli were applied through a pair of platinum electrodes resting on the dorsal surface of the animal. Condenser discharges from a 0·1 μF condenser were used. Frequency of stimulation was controlled by a hand key, metronome with mercury cups, or a commutator, in the discharge circuit. A minimal effective voltage was used, and this varied with the preparation (6–18 V.; usually 7·5 V. were effective).

OBSERVATIONS

Stimulation of the giant axons

Single discharges at intervals of 5 sec. to 2 sec. from a 0·1 μF. condenser, at constant voltage, produce single muscle twitches (Fig. 2*a*, *b*). At higher frequencies (1–2 stimuli per sec.) relaxation is incomplete between individual contractions and clonus results (Fig. 2*c*, *d*). At still higher frequencies (about 3 stimuli per sec.) typical tetanus ensues (Fig. 2*e*, *f*). These figures have relative significance only, since tetanus appears at lower frequencies of stimulation with onset of fatigue (Fig. 3). In the earthworm, Budington (1902) found that, with isotonic recording of longitudinal muscular contractions, tetanus developed at a stimulation frequency of 4 per sec.

It is characteristic of this animal that it is unable to maintain sustained contractions for long periods and progressive fatigue sets in, particularly at high rates of stimulation. The fatiguing process is reversible; after brief periods of rest the animal recovers the ability to contract during giant axon stimulation, but to a lesser degree than before. Longer periods of rest effect greater degree of recovery (Fig. 4).

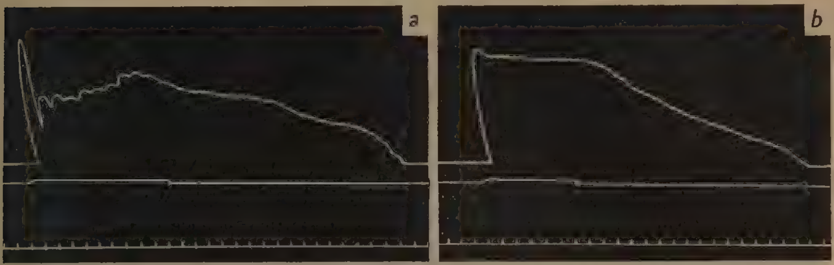

Fig. 3. Isotonic contractions, showing fatigue following repeated stimulation. Upper line, contractions of the animal; lower line, time scale at 1 per sec.; upward deflexion on middle line marks the duration of stimulation. Record *b* was taken immediately after *a*. Fatigue has greatly affected relaxation time, and resulted in fusion of separate contractions, but has not reduced the height of contraction.

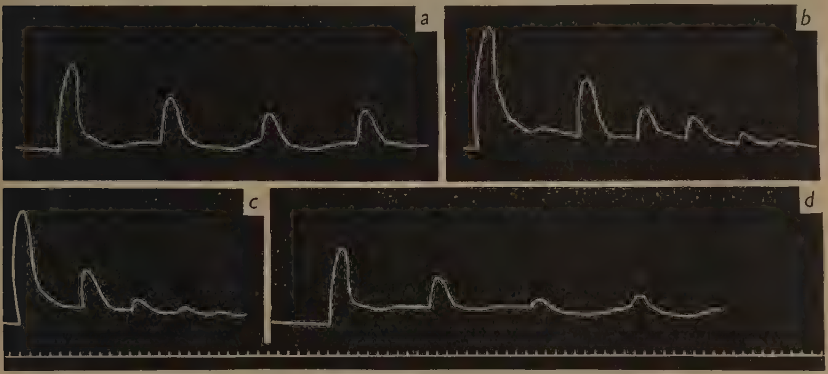

Fig. 4. Isotonic contractions at high frequencies of stimulation. Records taken in order shown. Time scale below, 1 per sec. Stimulation frequencies: *a*, 27 per sec.; *b*, 40 per sec.; *c*, 50 per sec.; *d*, 13 per sec. Muscle stimulated to complete fatigue on each occasion. About 5 sec. between successive stimulations.

Isotonic twitches frequently show an interesting delayed effect in that the lever does not immediately return to base-line, but the descent is delayed by secondary prolonged contractions (Fig. 2*a*, *b*, *c*, *d*). The tension developed by these secondary contractions is rather low, since they do not show in isometric records. They are apparently the result of enhanced tonic contractions in the longitudinal muscles following giant axon stimulation. An analogous process occurs in *Myxicola infundibulum* in that quick isotonic twitches are followed by periods of slow elongation (Nicol, 1948*c*).

Time course of contractions

Little information is available concerning the time course of contractions in annelid somatic muscle. Data are here presented for contractions in the longitudinal musculature following giant axon stimulation. Single twitches were recorded with a semi-isometric lever (on rather resistant steel-wire). Stimuli, by condenser discharges, were delivered at various rates; in Fig. 5 stimuli were 82 and 164 per min. consecutively, and the records show the progress of fatigue.

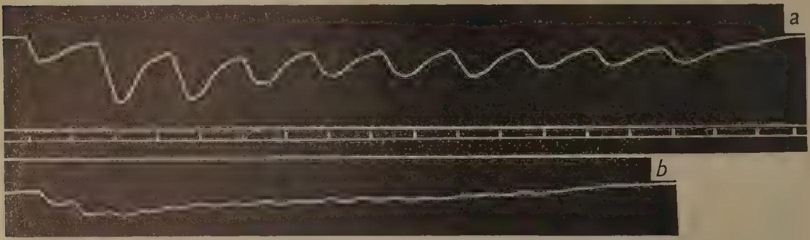

Fig. 5. Isometric contractions. Time scale, 1 per sec. Stimulation frequencies: *a*, 82 per min.; *b*, 164 per min. Effects of fatigue shown in extension of relaxation time, and reduction in tension developed.

Single contractions or twitches resulting from giant axon stimulation are fairly rapid. Maximal tension in this experiment (stimulation rate 82 per min.) developed 255 msec. after the beginning of contraction. Relaxation required about 1·8 sec. for completion. After 10 stimuli at 82 per min. the time course of contraction was considerably prolonged, maximal tension being attained after 475 msec., and relaxation being completed after about 3 sec. In *M. infundibulum* (another sabellid worm), single isotonic twitches were found to occupy about 2 sec., which result agrees well with the observations made upon *Branchiomma* (Nicol, 1948*c*). Budington (1902) has reported closely similar fatiguing effects in the longitudinal muscles of the earthworm after repetitive stimulation.

Effects of repetitive stimulation on contractions of the longitudinal muscles

In different groups of invertebrates there is considerable variation in the responses of muscles to repetitive stimulation. Characteristically each muscle fibre, when effectively excited, responds by an all-or-nothing contraction. In vertebrate striated muscle a single nervous impulse activates all the muscle fibres in that neuro-motor unit. A graded response results from the activity of an increasing number of neuro-muscular units. Comparable neuro-muscular studies of many invertebrate groups are still to seek. Thus Prosser & Young (1937), in a physiological investigation of the stellar nerves supplying the circular muscles in the mantle of the squid, found that there is normally no facilitation at the junction between the endings of the giant fibres and the muscles. Increased frequency of stimulation produces no increase in tension. They concluded that all the circular muscle fibres are activated by each nervous impulse reaching them. Some crustacean striated muscles are noteworthy, on the other hand, in that a single nervous impulse often produces no response. A series of impulses, however, causes contraction, and increase in frequency of stimulation often increases the mechanical tension developed by the muscle (review of extensive literature in Scheer, 1948, and Welsh and Schallek, 1946). Neuromuscular facilitation has also been reported in coelenterates (pertinent papers reviewed by Scheer, 1948, and Prosser, 1946). In *Calliactis*, for example, a single stimulus is inadequate; repetitive stimulation initiates contraction and with each successive stimulus the contraction increases (Pantin, 1935). Against this background it is of some interest to discover the character of the mechanism that occurs in the giant fibre system of polychaetes, as exemplified by *Branchiomma*. The simplicity of the giant axon-longitudinal muscle organization, and knowledge of the histological picture, permit an investigation in this species.

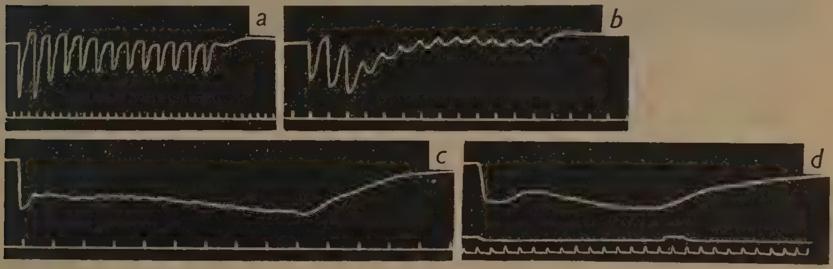

Fig. 6. Isometric contractions. Time scales, *a*, *b*, *c*, 1 per sec.; *d*, 5 per sec. Stimulation frequencies: *a*, 34 per min.; *b*, 64 per min.; *c*, 116 per min.; *d*, 13 per sec.

A typical experiment with isometric contractions is shown in Fig. 6. Contractions are represented by downward deflexions of the lever in these records, which are shown in the order obtained. At a stimulation frequency of 34 per min. there is complete relaxation between contractions in this experiment. At a stimulation frequency of 64 per min. complete relaxation between separate contractions occurs for the first two stimuli, after which some fusion takes place as the muscle fatigues and relaxation time increases. Fusion becomes more complete at higher frequencies (above 2 per sec.). There is no indication of increase in tension in these records with increase in frequency of stimulation; in fact, as stimulation is prolonged, tension soon falls to zero as fatigue sets in.

A few records taken with a semi-isometric lever show some increase in tension as the frequency of stimulation is increased in the range of partial relaxation. Examples are shown in Fig. 7. Records *a*–*e* are tracings taken in that order from one specimen;

f is an isolated record from another specimen. These results are ascribed to viscosity changes in the muscle with progress of fatigue. Budington (1902) similarly found an increase in the height of isotonic twitches in earthworm muscle under rapid stimulation. This increase in contraction paralleled, and resulted from, fatigue and prolongation of relaxation periods.

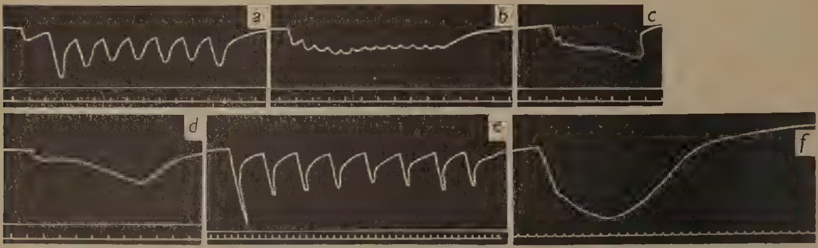

Fig. 7. Semi-isometric contractions. Time scales, *a*, *b*, *c*, *d*, *e*, 1 per sec.; *f*, 5 per sec. Stimulation frequencies: *a*, 36 per min.; *b*, 82 per min.; *c*, 164 per min.; *d*, 164 per min.; *e*, 12 per min.; *f*, 13 per sec.

The effect of increased tension on muscular elongation

Branchiomma is noteworthy in the amount of elongation it can undergo. When the entire animal is subject to stretching by gradual weighting, increase in length amounts to about 100% before the tissue ruptures (increases from an initial length of 12–26 cm. are representative figures for a mature animal). The records show certain peculiarities (Fig. 8). Initially, in all records, there are small increases in length as equal weights are added. Then at some period there are one or two steps of great increase in length, followed by a period of greatly reduced increments with increased loading. Deviation from true elasticity is shown by the partial return to original length (Fig. 8*b*). A strip of body wall, consisting predominantly of longi-

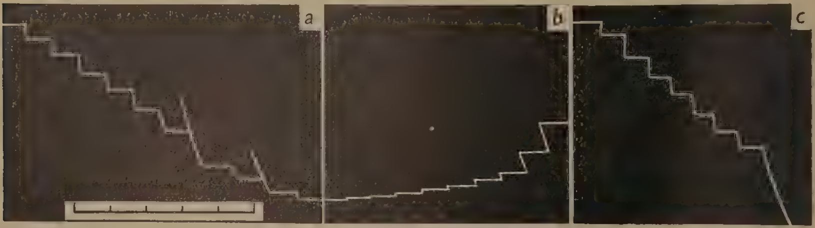

Fig. 8. Extension under loading. *a*, extension of the entire animal; *b*, shortening of the entire animal after extension; *c*, extension of a strip of body wall lacking nerve cord. The scale represents 5 cm. in actual length on the animal. Upward deflexions in *a* are due to contractions of the worm, stimulated by stretching. Other experiments in which contractions did not occur gave similar records. At the last load increment in *c* the tissue strip ruptured. Weighting in *a* was by successive additions of 1·6 g.; in *c*, by 0·8 g. In *b* the load was progressively reduced by 1·6 g.

tudinal muscle and lacking nerve cord, shows equal increments on graduated loading up to the point of rupture (Fig. 8*c*). The explanation advanced for the behaviour of the entire animal under tension is that tonus of the longitudinal muscles, controlled by the central nervous system opposes some degree of external tension. When the latter exceeds the internal force the muscle is stretched to the full extent commensurate with the load, and further weighting brings about smaller increments in length, in the expected deviation from Hooke's law.

DISCUSSION

The results of these experiments show that the neuro-muscular system comprising giant axons and longitudinal muscles functions in an all-or-nothing manner. A single excitable stimulus to the axons sets up a maximal muscular response, and further increments in frequency are ineffective in increasing tension when the muscle is not markedly fatigued. The histological arrangement of the giant axons indicates that an impulse, originating anywhere in either fibre, will proceed throughout the length of the body, spread out into the motor branches, and excite all the longitudinal muscles. The only synapse in the efferent side of this system lies at the neuro-muscular junction. Presumably some mechanism exists for the two giant axons to discharge synchronously, but their relationship to one another requires further study.

The structure and functioning of this system permit an interpretation in terms of the habits of the animal. The giant axons are involved in a withdrawal reflex in which the animal suddenly contracts and jerks back into its tube. Only one synergic contraction is involved normally in this response, which is maximal; a variable period of at least several minutes elapses before the animal emerges again and can be stimulated to contraction once more. Similar quick contractions attendant upon giant axon stimulation have been noted in *Myxicola infundibulum*, in which only one giant fibre is involved. Here it was found that increasing strength of stimulation, above threshold, is ineffective in increasing the extent of contraction, and contractions are all-or-nothing in nature. The systems in *Branchiomma* and *Myxicola* are suitable for sudden and rapid movements; apart from overcoming the inertia of the animal and building up hydrostatic pressure in the tube, little tension need be developed. The infrequency of stimulation in the normal life of the animal excludes the muscle fatigue observable under experimental conditions, and this contingency is doubly ensured by rapid adaptation in sensory pathways (Nicol, 1950).

An analogous situation has been investigated in the giant axon system of the squid (Young, 1937; Prosser & Young, 1937) in which a maximal response produced by single giant axon impulses serves to produce a sudden synergic contraction of the mantle and expulsion of a water jet in an all-or-nothing manner.

Obviously the functioning of the giant axons of *Branchiomma* is correlated with other neuro-muscular systems in the animal, but the exact relations still await exploration. It is probable that segmental connexions exist between the giant axons and motoneurones of the circular muscles, thereby affecting circular muscle tone during longitudinal muscle contraction. Other connexions possibly exist with nervous circuits serving septal and parapodial muscles in order to effect proper co-ordination of movement. Finally, some evidence is available that longitudinal muscle tone itself may be augmented following giant axon stimulation. A suggested explanation is that the giant axons secondarily discharge into segmentally arranged motoneurones that control localized responses.

The longitudinal muscles of *Branchiomma*, like those of other polychaetes, have striking features which distinguish them histologically from those of higher animals. In their absence of striation they can be classified as smooth muscle but they are more akin to striated muscle in their contractile properties. Physiological differences between muscles are probably quantitative, however, and these muscles of *Branchiomma* functionally occupy an intermediate position between the two extremes. It may be noted that in *Branchiomma* (and in *Myxicola*), synergic contraction of the animal stops at the level where the giant axons and nerve cord are cut. No evidence is thereby afforded for a syncytial muscle structure, in which a muscular excitation passes uninterrupted throughout the tissue as in vertebrate cardiac muscle. However, this is not to gainsay that whereas the sarcoplasm may be syncytial in nature the contractile filaments may still be discrete. Moreover, the transmission of a muscle potential has still to be demonstrated before this objection can be considered valid. It is worthy of note, in this connexion, that nervous rather than muscular transmission has been postulated for the intramuscular spread of excitation in insects (Roeder & Weiant, 1950). Further studies of neuromuscular physiology in polychaetes are obviously required.

SUMMARY

Synergic contractions following giant axon stimulation in *Branchiomma vesiculosum* have been investigated by the graphic method of recording. Isotonic and isometric levers were used, and electrical stimuli from condenser discharges were applied to the surface of the animal.

Single muscle twitches occur at stimulation frequencies up to 2 per sec., above which clonus, and finally tetanus result. At high rates of stimulation fatigue rapidly sets in; this fatigue is reversible.

Data for the time course of contractions are presented. Maximal tension develops about 255 msec. after the beginning of contraction, and relaxation occupies about 1·8 sec.

Maximal tension developed isometrically under stimulation at different frequencies (12 per min. to 13 per sec.) was measured. Maximal tension is developed initially, and there is no evidence for facilitation.

Extension of the animal and of strips of the body wall under tension are described.

The results are discussed in terms of the habits of the animal, and compared with similar studies of other invertebrates.

REFERENCES

BOZLER, E. (1936). *Symp. quant. Biol.* **4**, 260.
BRUNOTTE, C. (1888). Recherches anatomiques sur une espèce du genre *Branchiomma. Trav. Sta. Zool. Cette, Nancy.*
BUDINGTON, R. A. (1902). *Amer. J. Physiol.* **7**, 155.
HANSON, J. (1948). *Quart. J. Micr. Sci.* **89**, 139.
JOURDAN, E. (1887). *Ann Sci. nat.* **2**, 7e sér. 239.
NICOL, J. A. C. (1948*a*). *Quart. Rev. Biol.* **23**, 291.
NICOL, J. A. C. (1948*b*). *Quart. J. Micr. Sci.* **89**, 1.
NICOL, J. A. C. (1948*c*). *Canad. J. Res.* **26**, D, 212.
NICOL, J. A. C. (1948*d*). *Trans. Roy. Soc. Can.* **42**, v, 107.
NICOL, J. A. C. (1950). *J. Mar. Biol. Ass. U.K.* **29**, 303.
PANTIN, C. F. A. (1935). *J. Exp. Biol.* **12**, 119.
PRENANT, A. (1929). *Arch. Zool. exp. gén.* **69**, fasc. 1, 1.
PROSSER, C. L. (1946). *Physiol. Rev.* **26**, 337.
PROSSER, C. L. & YOUNG, J. Z. (1937). *Biol. Bull. Woods Hole*, **73**, 237.
ROEDER, K. D. & WEIANT, E. A. (1950). *J. Exp. Biol.* **27**, 1.
SCHEER, B. T. (1948). *Comparative Physiology.* New York and London.
WELSH, J. H. & SCHALLEK, T. W. (1946). *Physiol. Rev.* **26**, 447.
YOUNG, J. Z. (1937). *J. Exp. Biol.* **15**, 170.

THE DEVELOPMENT OF MOTOR RESPONSES IN THE STOMACH OF THE FOETAL SHEEP

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Knowledge of the movement of the gut in the mammalian foetus is mainly derived from observations on small laboratory animals. Yanase (1907) made a systematic study of pre-natal intestinal movement in the guinea-pig, and observed that the appearance of peristalsis coincided with the time of development of the longitudinal muscle of the gut, at about the 27th day of gestation. At the stage when circular muscle alone was present, he was able to elicit only local constriction by mechanical or faradic stimulation. Foetal gastric movement was observed in the rabbit (Tani, 1927) and in the cat by Friedman (1934), Windle & Bishop (1939), and Becker & Windle (1941). The last-named described rhythmic and persistent gastric peristalsis in cat foetuses of 35–40 days. Becker, Windle, Barth & Schultz (1940) found that thorotrast injected into the amniotic sac of the guinea-pig circulated through the alimentary tract of the foetus from the 42nd day onwards; and human foetal swallowing is said to occur from the fifth month (Windle, 1940). While there is evidence, therefore, that alimentary tract movement commences in pre-natal life, nothing is known of the factors controlling the onset of movement or of the nature of the individual movements.

The movements of the stomach of the foetal sheep are described here, as this species might be expected to yield more evidence than others concerning the spontaneous occurrence of movement, the nature of the movement, and the part played by the vagus in initiating it, for in adult ruminants the movements of the parts of the stomach are characteristic and marked. Further, Barcroft & Barron (1939) observed swallowing and suckling movements in the foetal sheep, and as suckling in the lambs is associated with a reflex closure of the structure known as the oesophageal groove, the onset of the reflex in foetal life can be expected to provide some index of the post-natal activity.

EXPERIMENTAL

Scottish Blackface and Dorset Horn ewes were used. Tupping dates were recorded and pregnancy was confirmed radiologically. With the Blackfaces radiological diagnosis of pregnancy was usually successful after 60 days, but it was less successful in the larger Dorset ewes at that age. This method enabled ewes with twin lambs to be selected when required.

The supply of material was planned so that foetuses from 40 days onwards could be obtained at ages increasing by 5–10-day intervals until full term. Table 1 gives the details of the stages selected.

Table 1. *The sheep used and the foetal ages at which the experiments were performed*

Ewe no.	No. of foetuses	Foetal age (days)	Ewe no.	No. of foetuses	Foetal age (days)
866	1(+1)*	40	694	2	*c.* 74
862	2	41	693	2	80
690	2	49	689	2	81
705	2	50	692	2	90
867	2(+1)*	50	695	2	100
711	1	*c.* 54	706	2	100
861	2	55	700	1	110
863	1	60	697	2	120
708	2	60	865	1	120
702	2	70	868	2	131
704	2	70	710	1	140

* 1 foetus carried to full term.

The ewes were given urethane intravenously in 25% solution until they were unable to stand. 'Duracaine' was then injected into the subarachnoid space in the lower lumbar region. Further urethane was given as necessary.

Nos. 861, 862, 863, 866 and 867, all Dorset Horns, were anaesthetized with 'Nembutal'. Caesarean section and the experiment on the foetus were performed under aseptic conditions, and the mother was allowed to recover. As these experiments were all performed during the early stages of gestation, anaesthetic effects upon the foetus were not observed. Nos. 866 and 867 had twin and triple foetuses respectively, one of which in each ewe escaped notice, and each ewe bore at full term, without assistance, a normal, healthy lamb. Hence surgical interference with the uterus as early as the 2nd month does not necessarily lead to abortion. In the case of non-recovery experiments the ewe was placed on her back in a bath filled with warm saline, in which the foetus was immersed, on Caesarean section being performed through the mid line.

The following observations were made on the foetus:

(*a*) presence or absence of swallowing or suckling;
(*b*) presence or absence of spontaneous gastric movement;
(*c*) the results of vagal stimulation;
(*d*) histological examination of the stomach.

(*a*) Spontaneous swallowing was studied in only five foetuses. The head of a foetus was located, a small incision was made in the uterine wall, and about 5 ml. of Indian ink was injected into the amniotic sac. The hole in the amnion was then quickly tied off, the uterine incision was sutured, and the uterine horn returned to the abdominal cavity, in an effort to disturb the foetus as little as possible while the twin was examined.

In studying induced swallowing, an incision was made in the uterus and the mouth was brought up to it. Barium sulphate suspension was dropped into the mouth, the rounded tip of the pipette being placed on the tongue in order to stimulate suckling. For the largest foetuses a finger was placed in the mouth and gently moved on the tongue. Foetuses were radiographed immediately after the end of the experiment, i.e. about 30 to 60 min. after the barium was given.

(*b*) and (*c*) After observing swallowing the foetus was drawn from the membranes into the bath, and supported so that the umbilical cord was free from tension. The stomach was exposed by removing most of the abdominal wall on the left side, making visible the rumen, reticulum and abomasum, but not the omasum. The left cervical vagus was exposed, but was not immediately cut.

Observations could then be made upon:

(1) the presence or absence of spontaneous gastric movement before stimulation;

(2) the effects of direct electrical stimulation of the stomach wall; and

(3) the effects of electrical stimulation of the uncut vagus, or of the central and peripheral ends separately, after the vagus was cut.

The observations were not always made in the same sequence. Faradic stimulation was supplied by a Palmer's induction coil with a pair of platinum wire electrodes. A constant coil distance of 10·5 cm. was used, at which a mild shock could be felt on the tongue. When observations on gastric movement were concluded the umbilical cord was tied off and the foetus removed and radiographed. In the foetuses used to study spontaneous swallowing the stomachs were opened to see where ink-stained fluid could be found. Before fixation each foetus was weighed and the crown-rump length measured as a check on the stage of gestation.

RESULTS

(1) *Spontaneous swallowing* occurred in each of five foetuses of between 90 and 120 days. No information was gained as to the oesophageal reflex, the ink always being diffused throughout the stomach.

(2) *Stimulated swallowing*. The 50–60-day foetuses responded to stimulation by slight movements of the tongue and jaws, but no throat movements were seen. Foetus 702A, of 70 days' gestation, was seen to open its mouth before the amnion was ruptured. When the pipette was placed in the mouth the tongue curled up at the sides, and throat movements were clearly seen. No contrast material reached the stomach. 704A and B, also 70 days, showed much the same degree of activity, but without the tongue movement. 694A was approximately 74 days. The mouth was closed round the pipette and the tongue curled slightly, and although swallowing movements were not pronounced, a small quantity of barium was found in the reticulum. 693B, 80 days; 689A, 81 days; 692B, 90 days; and 695B, 100 days, all suckled, closing their mouths on the pipette and swallowing, and with one exception barium was later found in the reticulum and rumen. 689A received 'Diodone', iodine contrast-fluid, instead of barium, and the radiograph, taken 30 min. later, showed the liver to contain a quantity of radio-opaque material, while the stomach was not shown up. Analysis of the liver gave an iodine content of 0·009 g.% (wet weight), while the control (the liver of the twin foetus) contained only a trace ($< 1\ \gamma$). This indicates rapid absorption from the stomach, but it is not possible to say with certainty from which part as, although at this age foetuses swallowed barium into the rumen and reticulum, the more diffusible suspension of Indian ink was found in the 90-day foetus to reach all four divisions. Of the six foetuses between 100 and 131 days none suckled as vigorously or swallowed as readily as those in the previous

3 weeks of development. Only two swallowed any barium; in 700A, 110 days, the barium was found in the rumen, reticulum, and body of the abomasum, which last position might indicate the appearance of an oesophageal reflex. Barium was also found mainly in the abomasum of the 131-day foetus. Finally, the 140-day foetus was active and suckled well (this ewe received only 15 g. of urethane instead of the normal dose of about 25); most of the barium passed to the abomasum, a small quantity going to the reticulum, and only a trace into the rumen.

706 A, 100 days, was used to investigate spontaneous swallowing of amniotic fluid, so it was not given barium, but after removing its head from the fluid to prevent further swallowing, it was offered a finger to suck. This foetus showed a well-developed pattern of suckling behaviour, closing its mouth firmly on the finger and waggling its tail.

(3) *Spontaneous stomach movement.* Spontaneous movement was not often seen on opening the abdomen, but it must not be assumed that such movement was normally absent, as in the adult it is inhibited by the procedure of laparotomy. It was never seen before 70 days, but at this age slight movements of the rumen were seen in one foetus, and intestinal motility in another.

(4) *Direct stimulation.* Direct electrical stimulation had no visible effect until 54 days, at which time the ventral sac of the rumen contracted but the response did not spread to any other part. In 55-day twins, stimulation of the ventral rumen sac produced a contraction spreading to the dorsal sac. The dorsal sac also responded to direct stimulation, but this response did not spread.

Table 2. *The first appearance of motor responses in the foetal stomach of the sheep*

Foetal age (days)	Response to	
	Direct stimulation	Peripheral vagal stimulation
40–50	0	0
54 approx.	Ventral rumen sac	0
55	Dorsal and ventral rumen sacs	0
60	Rumen sacs and localized reticulum contraction	Ventral rumen sac
70 onwards	All parts	All parts

At 60 days both rumen sacs responded separately. On one occasion the reticulum also showed a sluggish and incomplete local response similar to a rumen contraction; again a circular constriction of the intestine followed local stimulation. At 70 days stimulation of the rumen sacs still had only a localized effect, but stimulation of the reticulum caused a sharp reticulum contraction, followed slowly by a tetanic contraction of the ventral rumen sac, and sometimes by slow contraction of the body of the abomasum. Stimulation of the abomasum itself caused only a limited local response. From this age onwards local responses were usually strong in all parts of the stomach, but their spread from one part to another was always uncertain, and after 100 days it almost ceased. In one 100-day foetus the omasum was directly stimulated, but without any visible effect.

(5) *Vagal stimulation.* The earliest stage at which the cervical vagus could be

dissected out was 55 days, and it was then so delicate that no attempt was made to cut it. Stimulation was carried out by applying the electrodes to the intact nerve, and no response was detected. At 60 days stimulation of the intact nerve caused only a slight contraction of the ventral rumen sac.

At 70 days and onwards the vagus was cut in the neck and a sufficient length was freed along each stump to allow it to be laid over the electrodes. Stimulation of the peripheral stump at all stages from 70 days onwards always elicited immediate total contraction of the reticulum. It usually brought about more slowly, and with a latent period, tetanic contraction of the rumen, especially strong in the ventral sac. This response was less constant than that of the reticulum.

The ventral rumen sac contractions, from their first appearance, were spiral. They caused the left side of the sac to become elevated into a peak, so that the shape of the sac came to resemble that of a top-shell (*Gibbula*). Once this contraction had appeared it often persisted for some minutes. After several stimulations it tended to become stronger and more persistent, and sometimes remained visible after the death of the foetus. The effect did not appear after 100 days.

In early stages the effect of peripheral vagal stimulation on the abomasum was usually to produce tetanic contraction of the body of the organ. At other times stimulation was followed by strong peristalsis of the whole abomasum, but this did not always occur, and on one occasion relaxation was seen. This irregularity is illustrated in the following extract from a protocol:

Ewe no. 193, *2nd foetus*, 80 *days*

Peripheral stump of left vagus, first stimulation, 5 sec.

5 sec. Reticulum contracting strongly.

12 sec. Reticulum now relaxed. Strong contraction of whole body of abomasum.

15 sec. Ventral rumen sac beginning.

30 sec. Ventral rumen sac stronger. Pyloric antrum showing strong waves.

Two min. elapsed. Tonic contraction of abomasal body still present. Second stimulation, 5 sec.

5 sec. Reticulum contracted.

7 sec. Reticulum relaxing. Upper end of abomasum rather suddenly relaxed, but contraction persisted in a band at the 'antral furrow'.

25 sec. Ventral rumen sac contracting.

35 sec. Abomasal peristalsis recommenced.

Stimulation of the central stump of the cut cervical vagus was not productive of very definite results on the stomach, except that it was never followed by total contraction of the reticulum.

Contractions of the individual sacs of the rumen, of the abomasum, and surface movements on the reticulum were sometimes produced, and a spasmodic movement of the diaphragm often occurred.

In two foetuses only, at 120 and 131 days, the right cervical vagus was also cut after the left, and the effects were then studied of direct stimulation of the reticulum. In one, strong partial contractions involved about half the reticulum, but never the whole organ, although stimulation of the peripheral vagal stump still produced total contraction. In the other, similar partial contractions were brought about by stimulation of the anterior and dorsal part of the organ, but stimulation of the lower pole or of the posterior border against the anterior rumen sac gave practically a complete contraction. In fact, the results of direct stimulation seemed to be much the same in the presence or absence of the intact vagus.

(6) *Other observations.* The abomasum and top of the duodenum were seen on four occasions to be extremely active and the activity was analysed. The strong movement was never seen until some stimulation had been given, but a single vagal stimulation, either central or peripheral, as if ending an inhibition, often induced rhythmic movements which continued until the end of the experiment, except when modified by further stimulation. The movement seen in these foetuses was particularly interesting, as it has not hitherto been recorded in the adult sheep. Peristaltic waves passed down the abomasum in the typical manner; comparatively few waves passed over the body of the organ, although those which did so originated near the omaso-abomasal junction and passed right down to the pylorus. In between these, other waves originated at the incisura angularis and passed along the pars pylorica. Every third or fourth wave reaching the pylorus, whether it came from the top of the abomasum or not, was followed by a sudden contraction or short series of contractions of the pylorus and the visible part of the duodenum (about the first 1 or 2 cm.). Several of these contractions might occur in rather rapid succession, with intervals of four or five seconds. Each contraction seemed to occlude the lumen completely, and the wall was blanched, so that the appearance resembled that of a cardiac ventricular beat. It is therefore referred to as the duodenal beat. An example of the time intervals between successive beats, in seconds, is: 35, 15, 5, 15, 20, 5, 5, 20, 5, 40. The beating seemed to be related to the state of filling of the pyloric antrum. In two foetuses where the antrum was not distended single beats were seen to follow vagal stimulation. Altogether, single or multiple beats were seen to occur from 90 days to full term.

HISTOLOGY

Attempts were made in the first few experiments to perfuse foetuses with methylene blue by way of the umbilical vein, but this was abandoned. Satisfactory general views of the myenteric plexus were obtained from 60 days onwards by Gairns's gold chloride impregnation; this was less satisfactory in the earlier stages, possibly owing to a lack of differential metabolism in the nervous tissue. In the early stages the main fibre tracts and ganglia showed up quite satisfactorily in sections of material fixed in alcohol-formalin-acetic and stained with Heidenhain's haematoxylin.

A pair of 26-day embryos were obtained from a ewe which was a casualty; one was serially sectioned. No differentiation of muscle fibres was seen in the stomach, and no sign of nerves.

The next foetal stomach to be sectioned was one of 41 days' gestation. At this stage the inner, circular muscle layer was found to be differentiated, the main nerve trunks were present, and ganglia and tracts were seen outside the muscle layer.

By 50 days a few fibres of the outer, longitudinal muscle layer appear outside the nervous tissue, which is now much more prominent and is in fact most abundant in relation to the size of the organ. At 55 days there is quite a well-marked longitudinal muscle layer, and the proportion of muscle to nerve tissue grows rapidly greater from this point to the end of gestation.

DISCUSSION

Hamilton, Boyd & Mossman (1945) describe two stages in the development of motor mechanisms involving striated muscle, namely, a non-functional and a functional period. The latter is subdivided into a myogenic stage (when the muscle fibres can contract before the nerve supplying them can conduct), a neuromotor stage ('in which direct stimulation of the centre of the nerve trunk causes muscular contraction'), and finally a reflex stage. These authors point out that the 'three stages overlap to considerable extent, and some investigators (e.g. Straus & Weddell, 1940) think that the separation of a myogenic stage is artificial. Further, the reflex effects are not so localised as they are for a similar stimulus, in post-natal life.'

It is more difficult to recognize a reflex stage in smooth muscle, where autochthonous movement may occur in the absence of central control than in skeletal muscle, where an extrinsic stimulus is needed for contraction. Our observations have not provided definite evidence of the initiation of a reflex stage, except the appearance of the oesophageal reflex.

The earlier stages of functional development in the sheep's stomach appeared to be identical with those described by Hamilton *et al.* (1945). The non-functional period ended with the differentiation of the longitudinal muscle layer, between the 50th and 54th days of gestation. The myogenic stage had commenced by the 54th day, when the rumen was able to contract in response to direct stimulation. At 60 days the myogenic might be said to overlap the neuromotor stage, as movement of the ventral rumen sac could be elicited by vagal stimulation, while the dorsal sac and the reticulum were still in the stage of myogenic activity, brought about only by direct stimulation of the muscle. Although response both to direct and to vagal stimulation appeared first in the ventral rumen sac, no precocity in development of this part was detected histologically.

Whereas up to 60 days the only movements seen were direct responses to stimulation, from 70 days onwards sustained movements, such as successive contraction waves, were seen to occur. Vagal stimulation after this time affected the whole stomach, and the types of response to it resembled those of the adult stomach. Moreover, Barcroft (1946) first obtained evidence of vagal effects on the heart at 77 days from the right vagus, and at 81 days from the left. It seems likely that this stage, when adult responses are obtained, is akin to the development of the reflex stage in skeletal muscle. Further work would be necessary to discover whether the spread of contraction from one part of the stomach to another, which occurred at 70 days, involved nervous elements. Probably it was partly due to a simple spread of the electrical impulse across the small organ, and the decrease in spread after 100 days was a result of the increasing size of the stomach.

There was, however, one more constant finding which continued to full term, namely, that total contraction of the reticulum resulting from stimulation was nearly always followed, after a latent period, by peristaltic movement of the body of the abomasum.

Further work is required on the sensory aspect of the reflexes controlling gastric movement, especially in view of the varying results of stimulation of the central end of the vagus. The motor activity of the vagus is indicated by the results described above, showing that major movements are possible from approximately the time when the vagus becomes functional, and it has been confirmed by bilateral vagotomy in lambs, after which operation all major rumen and reticulum movements cease.

Suckling is a complex pattern and is built up over a long period of development. Responses of the mouth and tongue to touch stimuli were not seen until the 50th day, but Barcroft & Barron (1939) saw them as early as the 41st day, and they also saw 'throat movements...which indicated the foetus is sucking' at 49 days. We failed to detect them until the 70th day, by which time there was an external appearance as of sucking and swallowing, although the first foetus which actually swallowed was 4 days older. The ability to suck quite strongly was in evidence by 80 days, but it was 100 days before tail-wagging entered into the complex, and 110 days when there was any sign of oesophageal groove closure. According to Barcroft & Barron (1939) 'the effect of the urethane on the behaviour of the foetus becomes more marked as the age of the foetus increases'. As we were using urethane as an adjunct to the spinal anaesthesia, a full dose was not needed, but the foetuses were increasingly affected in the later stages. They were never anaesthetized, but were sluggish, and this accounts for the difficulty of obtaining suckling responses. Since the ewe only received 15 g. of urethane instead of 25–30 g., the oldest foetus of all, 140 days, was less affected, and it sucked readily, exhibiting a functional oesophageal reflex. Watson (1944) has shown the oesophageal reflex to be closely linked with the pattern of behaviour indicative of 'pleasure in suckling'. We have never actually watched the effect on the groove of vagal stimulation, but bilateral vagal section in suckling lambs abolishes the reflex, and this suggests that the vagus provides motor control for this as for other gastric movements. It is hoped to make a further study of the level of central nervous control and, if possible, the sensory arc.

SUMMARY

The state of motor activity of the stomach was studied in a series of foetal sheep. The non-functional period was found to end between the 50th and 60th days of gestation. Sustained activity was preceded by short periods of myogenic and neuromotor activity, and was not in evidence until the 70th day. Foetal swallowing commenced soon after this, but the pattern of suckling behaviour developed gradually over an extended period. The motor mechanisms mediated by the vagus were studied by section and electrical stimulation of the nerve.

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REFERENCES

BARCROFT, J. (1946). *Irish J. Med. Sci.* p. 603.
BARCROFT, J. & BARRON, D. H. (1939). *J. Comp. Neurol.* **70**, 477.
BECKER, R. F. & WINDLE, W. F. (1941). *Amer. J. Physiol.* **132**, 297.
BECKER, R. F., WINDLE, W. F., BARTH, E. E. & SCHULTZ, M. D. (1940). *Surg. Gynec. Obstet.* **70**, 603.
FRIEDMAN, M. H. F. (1934). *Proc. Soc. Exp. Biol., N.Y.*, **34**, 495.
HAMILTON, W. J., BOYD, J. D. & MOSSMAN, H. W. (1945). *Human Embryology*, p. 272. Cambridge: Heffer.
STRAUS, W. L. & WEDDELL, G. (1940). *J. Neurophysiol.* **3**, 358.
TANI, K. (1927). *Jap. J. Obstet. Gynec.* **10**, 2.
WATSON, R. H. (1944). *Bull. Coun. Sci. Industr. Res., Aust.*, no. 180.
WINDLE, W. F. (1940). *Physiology of the Fetus.* Philadelphia and London: Saunders.
WINDLE, W. F. & BISHOP, C. L. (1939). *Proc. Soc. Exp. Biol., N.Y.*, **40**, 2.
YANASE, J. (1907). *Arch. ges. Physiol.* **117**, 345.

THE INTEGRATION OF ACTIVITY CYCLES IN THE BEHAVIOUR OF *ARENICOLA MARINA* L.

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(With Five Text-figures)

The common lugworm exhibits two distinct types of spontaneous activity cycle. Each of them has been described in some detail, but the method of approach was rather different in the two cases, and their integration has not yet been discussed. The experiments to be described were made to throw light on their mutual relations. The work was done at the laboratory of the Marine Biological Association of the United Kingdom, at Plymouth, in March and April 1950.

The relevant features of the two cycles, as hitherto described, will first be summarized. For a more detailed review see Wells (1950).

THE OESOPHAGEAL CYCLE (FEEDING, OR *f* CYCLE)

This rhythm has been studied mainly on dissected preparations.

The 'isolated extrovert' preparation consists of the proboscis with a short length of oesophagus attached. It gives bursts of vigorous rhythmic contraction alternating with periods of relative rest. The time taken by a whole cycle is generally 6–7 min. Single preparations are very regular in their timing, and the variation is chiefly from preparation to preparation. The intermittent pattern arises in a diffuse structure, probably a nerve plexus, in the wall of the oesophagus, and is transmitted to the powerful muscles of the proboscis, to whose activity the movements of the writing lever are mainly due (Wells, 1937).

The pattern can be shown to spread further, by way of the nerve ring and cord, to the muscles of the body wall, but the type of effect which it there produces varies in the different regions of the body. The body wall of the first three segments exhibits vigorous rhythmic contractions during the outbursts of the oesophagus, and there is close integration between the individual contractions in the extrovert and body wall (Wells, 1937). The body wall of the branchiate segments behaves quite differently; it traces a continuous and often very regular wave pattern, and this is usually inhibited, by reduction of amplitude, at each outburst of the extrovert. Sometimes—but by no means invariably—the body wall of the branchiate segments shows a vigorous and rather prolonged contraction as the oesophageal outburst passes off (Wells, 1949*b*).

When one has acquired the trick of making the dissections, these main results can be got with great regularity, especially during the cooler months of the year. Their interpretation in terms of the activities of the whole worm is rather tentative, and rests on four pieces of evidence.

(*a*) If intact worms are watched in glass tubes, the majority show cycles of activity of the anterior end, whose vigour is somewhat variable. They may appear as outbursts of extrusion and withdrawal of the proboscis, or merely of swaying, stretching and gulping movements. But in either case their timing agrees with that of the *f* cycles of the oesophagus, whose outward expression they therefore seem to be. It was suggested, from these observations, that the oesophageal pacemaker determines intermittent feeding under natural conditions (Wells, 1937).

(*b*) The feeding of the closely related species *A. cristata* Stimpson was described by MacGinitie & MacGinitie (1949, p. 202) in the following words: '...the process of everting and inverting the proboscis and then swallowing occurs about every 5 sec. Also, after a certain number of swallows, ranging from 8 to 15, the worm takes a rest period of a few minutes, then begins feeding again.' This account agrees very well with the behaviour of the dissected preparations of *A. marina*.

(*c*) The difference in response between the anterior segments and the branchiate ones corresponds to a functional distinction. The first three segments co-operate with the proboscis itself in bringing about the acts of extrusion and withdrawal. The branchiate segments perform wave movements, which travel along that part of the body in creeping and in the driving of water through the burrow (Just, 1924; van Dam, 1938).

(*d*) The behaviour of intact *A. marina*, living normally in sand, can be studied by recording the water movements through the burrow. The tracings often show a periodicity whose timing agrees with that of the *f* cycle. As the outbursts of the oesophageal pacemaker cause inhibition of the body segments responsible for irrigation, we may reasonably attribute these oscillations of the water movement trace to the influence of this pacemaker, and accept them as confirmation that the *f* cycle is not merely a property of the dissected preparations, but a rhythm of real importance under natural conditions (Wells, 1949*b*).

THE IRRIGATION-DEFAECATION CYCLE (*i-d* CYCLE)

In this case, the observations have mostly been made on intact worms.

The lugworm lives in a burrow of characteristic structure (Wells, 1945). If the water movements through a burrow are recorded, the tracing commonly shows long stretches, lasting for many hours, of very regular cyclic activity. Besides the oscillations mentioned above, there are conspicuous diphasic peaks, occurring at intervals of about 40 min. Each excursion is due to a backward movement of the worm to the surface of the sand, for defaecation, followed by a spell of vigorous headward irrigation, during which it creeps back to the bottom of its burrow. This type of tracing is not always obtained, but it occurs whenever the worm is feeding in the normal manner, at the base of a gradually subsiding funnel, and building up a pile of faeces. At other times, there may be other types of record, such as continuous, confused activity or complete rest; but when these appear, the worm is neither feeding nor defaecating (Wells, 1949*b*).

Identical cycles are often traced by worms in glass tubes, and it can be shown, by appropriate experimentation, that the excursions are not reflexly produced, either by the presence of faecal matter in the rectum, or by oxygen exhaustion or carbon dioxide accumulation. They are due to the activity of a second spontaneous pacemaker system (Wells, 1949*a*, *b*).

Very little has been done with dissected preparations, to locate the pacemaker for the *i-d* cycle, or to trace the spread of its pattern through the neuromuscular system. Records of the spontaneous behaviour of longitudinal body-wall strips from the branchiate part of the body suggest that the ventral nerve cord is the seat, or a necessary part, of the pacemaker (Wells, 1949*a*).

THE INTEGRATION OF THE TWO CYCLES

When considering the functional meaning of the *f*- and *i-d* cycles, the problem of their integration at once arises. One would suppose it hardly profitable, or likely, for the worm to indulge in an outburst of feeding while moving backwards to the surface for defaecation. Is there, then, an inhibition of either pacemaker by the other, by which such disharmonies can be avoided?

To explore this question, we set up a series of eighteen dissections, differing slightly in detail, but all on the general plan illustrated in Fig. 1. The worm is pinned ventral side downwards, and care is taken throughout the dissection to avoid injury to the nerve ring or cord. The extrovert is prepared as previously described (Wells, 1937). The oesophagus is cut across, and its oral end is connected to a light isotonic lever. A strip of circular muscle is taken from the more anterior segments and operates the second lever. The movements of the branchiate region are recorded by immobilising a couple of segments with pins passing through the neuropodia, and connecting a third lever to a fine hook passed through the dorsal body wall. Finally, the basal region of the tail is similarly recorded, though in this case, owing to the slenderness of the tail, the pinning is such that the longitudinal musculature takes a large part in determining the movements of the lever. The nerve cord was divided in the course of several of the experiments.

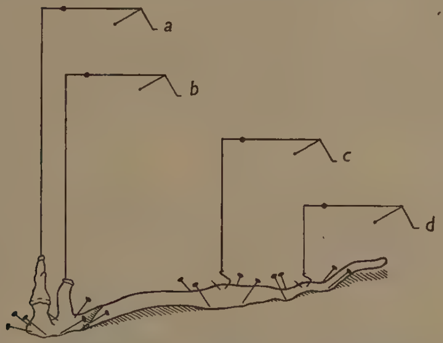

Fig. 1. Dissection for simultaneously recording the movements of the extrovert (*a*), the anterior segments (*b*), the branchiate segments (*c*) and the tail (*d*).

We used slowly moving drums (at 40–80 mm./hr.), because we were looking for signs of the long-period *i-d* cycle, and frontal writing points, to facilitate the analysis of the tracings. The pull on the preparation was 0·4 g. for each lever (see Wells & Ledingham (1942) for a note on the effect of lever tension).

The preparations were mounted in sea water, at temperatures ranging from 13 to 16·7° C. The salt balance of sea water is practically identical with that of *Arenicola* body fluid (Robertson, 1949). The isolated extrovert shows *f* cycles either in sea water or in body fluid (Wells, 1937).

The chief results can be seen in the extract of Fig. 2, which was traced by a preparation set up exactly as in Fig. 1. The following points are clear.

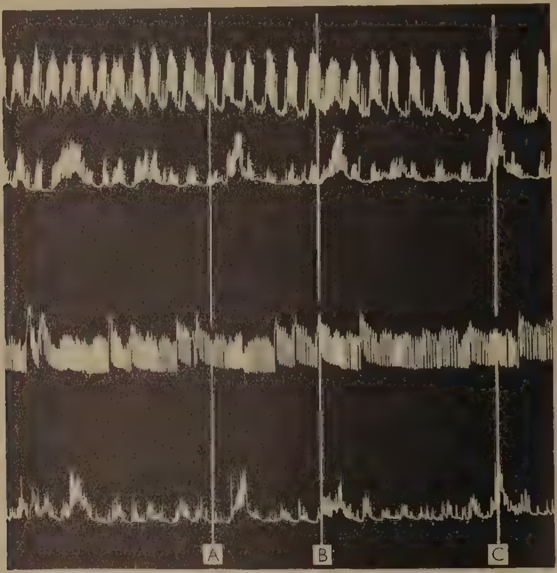

Fig. 2. Extracts from the record of an *A. marina*, set up as in Fig. 1. The traces are, from above downwards, extrovert, anterior segments, branchiate segments, tail. The extract is 4 hr. long, and begins 4 hr. after the start of the experiment. Read all records from left to right. (Worm A.M. 7.)

(*a*) The *f* cycle appears with great vigour and regularity on the extrovert trace. It is transmitted to the body wall and each outburst can be seen as a group of contractions in the anterior body-wall strip, and usually as a decrease in amplitude in the branchiate region. Line A marks a typical *f* cycle burst, showing these effects. On many occasions (e.g. at B) the decline of an oesophageal burst is accompanied by a vigorous contraction of the branchiate segments. Contractions of exactly similar appearance are often shown by the branchiate segments after division of the nerve cord between it and the extrovert, so they cannot be directly due to impulses arriving from the extrovert. Their correlation with the *f* cycle, in undivided preparations, is perhaps due to a 'rebound' period of raised excitability, as the inhibition caused by an oesophageal outburst passes off.

Records of the various effects of the *f* cycle, taken on faster drums, were published by Wells (1937, fig. 21; 1949*b*, figs. 6, 7). They have not previously been recorded simultaneously from a single preparation.

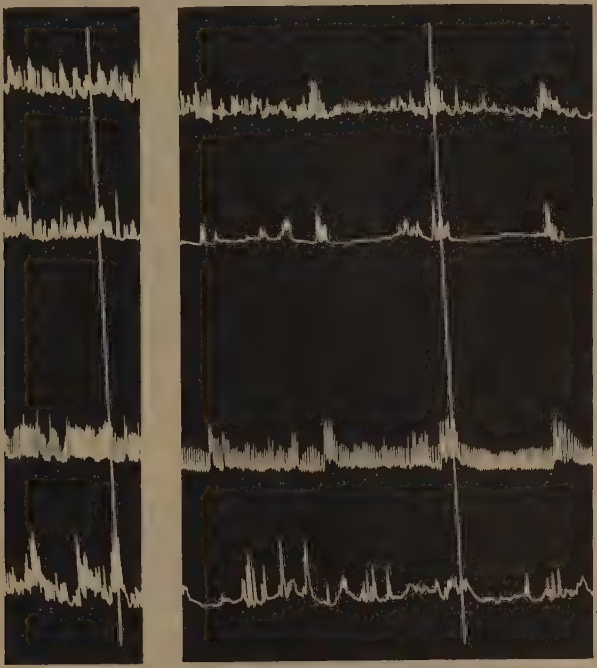

Fig. 3. *A. marina*, set up as in Figs. 1, 2. The first extract is 1 hr. long, and begins 2½ hr. after the start of the experiment. The second is 3 hr. long and begins 8 hr. after the start. The alignment of the levers in this experiment was not quite vertical, as shown by the sloping lines. (Worm A.M. 10.)

(*b*) Outbursts of another type appear very plainly, though at rather irregular intervals, in the lower three lines (e.g. at C). They are seen as vigorous spells of activity in the anterior body wall and tail, and as a marked acceleration in the branchiate segments. They have no effect at all on the extrovert trace; nor is there any reciprocal effect, for they may appear during either the outbursts or the pauses of the *f* cycle.

The second type of outburst has not previously been recorded. It was traced by twelve of the eighteen preparations in this series. We interpret it as the expression, by the dissected worm, of the *i-d* cycle. Our reasons are as follows.

(i) The intervals between the outbursts, though rather variable, are of the same order as in the case of the *i-d* cycle. The worm of Fig. 3, for example, gave bursts at intervals of 15–20 min. in the first part of the experiment, then the period lengthened out to about 50–55 min. (The appearance of the outbursts on the extrovert trace, in the second extract, is unusual, and is discussed below.) The interval is generally 20–60 min., but the range encountered in the whole series of experiments was 10 min. to 2 hr. The intact worm, living under favourable conditions in sand, makes defaecatory excursions about once every 40 min. It exhibits corresponding outbursts of activity in glass tubes, and they often occur very regularly at about the same interval. However, their frequency can vary; they are accelerated if oxygen is admitted after a period of oxygen lack; and they may also change their timing without discernible external cause. A rather extreme example of the latter type of variation is given in Fig. 4. The worm was put in a glass U-tube for 48 hr., and the water movements were recorded by the technique described elsewhere (Wells, 1949*a*). For 10 hr., on the afternoon of the first day, it gave a very regular series of bursts of pumping, at intervals of about 50 min. (upper extract). These, of course, are *i-d* bursts. There followed 5 hr. of continuous, but rather fluctuating, activity; then a period of *i-d* bursts at intervals of up to 2¼ hr. (second extract). The third extract is the direct continuation of the second, and shows a period of continuous, fluctuating activity, followed by *i-d* bursts whose intervals are at first short (15 min.) and then lengthen out again. There is in fact a close resemblance between the intact worms in glass tubes and the dissected ones, not only in the most usual intervals between the bursts, but also in the kind of variations that appear, and in the extreme values of the range.

(ii) Whole worms in glass tubes are generally motionless, or nearly so, between the irrigation outbursts. During the latter, the waves travel along their bodies at frequencies of the order of 8 waves per min. (van Dam, 1938). In sand, they pump water gently between the bursts, and the water movement trace often shows a periodicity suggesting inhibition of pumping by the *f* cycle; the *i-d* outbursts involve a great increase in the velocity of the stream (Wells, 1949*b*, figs. 3, 5). The branchiate segments of the dissected preparations show waves at about 1–2 per min. between the bursts, with amplitude inhibition by the *f* cycle. This frequency is too slow for vigorous irrigation, but it might produce the gentle water movement seen with worms in sand between the *i-d* outbursts. The bursts in the dissected preparations involve acceleration of the rhythm of the branchiate segments, and this is consistent with the suggestion that they represent *i-d* outbursts.

(iii) We have seen nothing else in the dissected preparations that could correspond with the *i-d* cycle; neither do we know of any other periodic activity in intact worms with which the bursts of the dissected ones could be equated.

The behaviour of the intact worm is of course variable. The *i-d* cycle is not always shown, and when it is, its frequency is modifiable, for example by previous oxygen lack. Similarly, the worm is capable of sustained spells of vigorous proboscis activity, for example when dug up and replaced on the surface of the sand. Nevertheless, it has a characteristic behaviour pattern, governed by the two cycles, into which it settles whenever conditions are favourable. According to our interpretation, the operated worms tend to settle into the same routine, showing an indifference to dissection which is indeed remarkable.

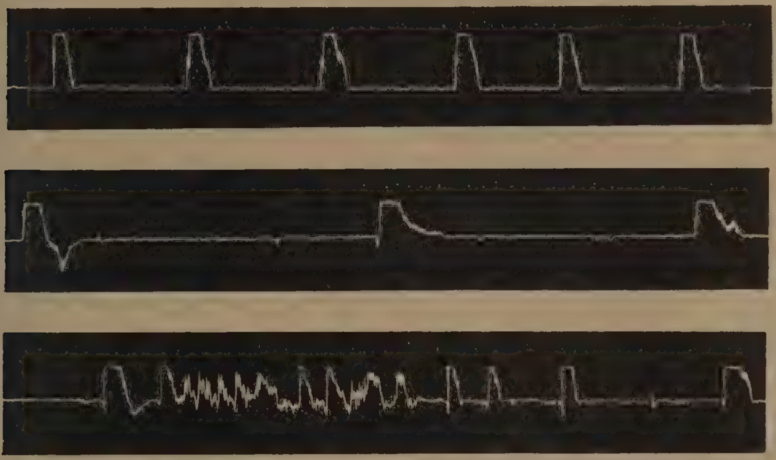

Fig. 4. *A. marina.* Three extracts from a record of the water movements set up by an intact worm in a glass tube. Upstroke of the lever means a headward propulsion of water. Each extract is 5 hr. long. (Worm I.M.)

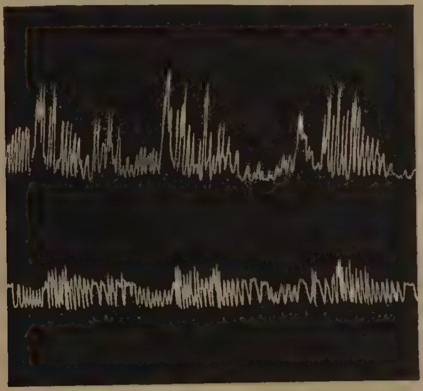

Fig. 5. *A. marina*, set up as in Figs. 1–3. The extract shows the movements of the anterior segments (above) and branchiate segments (below) after division of the preparation just behind the extrovert. The extract is 1 hr. 20 min. long, and begins 1½ hr. after the division was made. (Worm A.M. 6.)

The *i-d* cycle is sometimes traced by the body wall after complete isolation from the extrovert (Fig. 5).

According to our results, neither of the two pacemakers has any direct influence on the rhythm of the other. It will however be noted that the oesophagus has been cut across, when the experiment is set up as shown in Fig. 1. There is no reason to assume that the nerve cord is the only conducting path between extrovert and body wall; the two pacemakers might conceivably modify each other's activity by way of the gut. To test this possibility, we kept the oesophagus intact in a further series of experiments, and recorded the movements of the extrovert by means of a fine glass hook passed through one side of the oesophagus, at about the level of the septal pouches. The main result was the same as before. The *f* and *i-d* cycles showed no mutual reactions.

The activities evoked by the two pacemakers could be integrated, either by directly controlling the initiation of their respective outbursts, or by regulating the extent to which their influences spread through the neuromuscular system. Our results give no support to the first possibility, but there are the following indications that the second may occur.

(*a*) As already stated, the oesophageal rhythm may appear in the intact worm, either as vigorous proboscis action, or, with the same timing, as gentler acts (Wells, 1937). This presumably means that the number of muscle fibres reached by impulses from the oesophagus varies with conditions.

(*b*) The trace of the anterior body wall, in Fig. 2, clearly shows that it serves two masters. It follows the extrovert, in between the *i-d* outbursts, but when these appear, they take charge and dominate its behaviour. Similarly, the *f* cycles are commonly seen as periodic inhibition of the branchiate segments in the intervals between the *i-d* outbursts, but there is no evidence in our records that they have any inhibitory influence while the latter are in progress.

(*c*) In a few preparations, the *i-d* cycles could clearly be detected on the extrovert trace. An example is seen in the second half of Fig. 3. The extrovert of this preparation began quite vigorously, but, in the 8 hr. which elapsed before the beginning of the second extract, it gradually weakened and failed; the *f* cycle disappeared, first from the anterior body wall trace, then from that of the extrovert itself. At the same time, a spread of the *i-d* cycle into the extrovert trace occurred. The muscles responsible may be those of the proboscis itself, or those of the body wall round the mouth; for it is impossible, when setting up preparations of this type, to prevent a certain amount of invagination of the body wall of the head, which thus adds itself to the extrovert proper. In any case, however, a comparison of Figs. 2 and 3 shows that the extent of spread of the *i-d* cycle is variable.

The results rather suggest a competition for territory between the two pacemakers. It is, perhaps, by the physiological control of this competition, that their integration is achieved. In particular, if both pacemakers happen to discharge their outbursts simultaneously, the *i-d* cycle prevails over most of the body, and the *f* cycle in the region of the extrovert only. There is apparently nothing to prevent the occurrence of an oesophageal outburst during a defaecatory excursion; but as, at such a moment, practically the whole of the body wall would be under the domination of the *i-d* pacemaker, we may presume that the *f* outburst could only evoke extremely localized acts, such as internal movements of the proboscis and perhaps opening and closing of the mouth.

An incidental point was noticed during the experiments. In six of the ten experiments in which the nerve cord was divided, there was temporary inhibition of the hinder end of the worm immediately after the cut. In one experiment there was definitely no inhibition, while in three the point was doubtful. There was never any inhibition of the front end. These facts suggest that a condition may follow section of the cord, similar to the well-known spinal shock in vertebrates.

SUMMARY

1. Lugworms were dissected in such a way that the movements of the following parts could be simultaneously recorded: extrovert, body wall from the anterior three segments, body wall from the branchiate segments, tail. The preparations were set up in sea water and tracings were taken for many hours in each case. The preparations typically settled down to give cyclic behaviour patterns, remarkably similar to those which intact worms exhibit under favourable conditions, and in which two components were conspicuous.

2. The first, and most invariable, component is the feeding cycle (*f* cycle), of period 6–7 min. This rhythm originates in the oesophagus, and is transmitted to the muscles of the proboscis (where it causes outbursts of vigorous contraction) and body wall (where it causes correlated contractions in the first three segments, but periodic inhibition in the branchiate segments).

3. The second component was seen in two-thirds of the experiments. It consists of bursts of vigorous rhythmic activity in the body wall and tail, and can appear after their connexion with the extrovert has been severed. Under exceptional circumstances (exhaustion of the *f* cycle) it may spread to the extrovert trace. Its period is generally 20–60 min. It is apparently identical with the irrigation-defaecation cycle (*i-d* cycle) of intact worms.

4. Neither pacemaker directly affects the rhythm of the other. The integration of the activities which they determine probably depends on variation in the extent to which their influences spread through the neuromuscular system. They appear to compete for territory. If they happen to discharge outbursts simultaneously, the *i-d* pacemaker dominates over most of the body wall, and the *f* pacemaker over the proboscis and mouth region.

REFERENCES

DAM, L. VAN (1938). *On the Utilization of Oxygen and the Regulation of Breathing in some Aquatic Animals*. 143 pp. Groningen.

JUST, B. (1924). Über die Muskel—und Nervenphysiologie von *Arenicola marina*. *Z. vergl. Physiol.* **2**, 155.

MACGINITIE, G. E. & MACGINITIE, NETTIE (1949). *Natural History of Marine Animals*. 473 pp. New York.

ROBERTSON, J. D. (1949). Ionic regulation in some marine invertebrates. *J. Exp. Biol.* **26**, 182.

WELLS, G. P. (1937). Studies on the physiology of *Arenicola marina* L. I. The pacemaker role of the oesophagus, and the action of adrenaline and acetylcholine. *J. Exp. Biol.* **14**, 117.

WELLS, G. P. (1945). The mode of life of *Arenicola marina* L. *J. Mar. Biol. Ass. U.K.* **26**, 170.

WELLS, G. P. (1949*a*). Respiratory movements of *Arenicola marina* L.: intermittent irrigation of the tube, and intermittent aerial respiration. *J. Mar. Biol. Ass. U.K.* **28**, 447.

WELLS, G. P. (1949*b*). The behaviour of *Arenicola marina* L. in sand, and the role of spontaneous activity cycles. *J. Mar. Biol. Ass. U.K.* **28**, 465.

WELLS, G. P. (1950). Spontaneous activity cycles in polychaete worms. *Symp. Soc. Exp. Biol.* no. IV, pp. 127.

WELLS, G. P. & LEDINGHAM, I. C. (1942). Studies on the physiology of *Arenicola marina* L. III. The potassium relations of the isolated extrovert (with remarks on the effects of varying the lever weight). *J. Exp. Biol.* **19**, 176.

THE ROLE OF OESOPHAGEAL RHYTHMS IN THE BEHAVIOUR OF *ARENICOLA ECAUDATA* JOHNSTON

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(*Received* 24 *June* 1950)

(With Three Text-figures)

The British fauna includes three species of *Arenicola*. The most abundant is the common lugworm, *A. marina* L., which lives in great numbers on tidal sand flats and mud flats. The other two, *A. ecaudata* Johnston and *A. branchialis* Aud. & Edw. (= *A. grubii* Claparède), are found, usually together, in coarse, rather foul, sandy material, between stones and rocks.

A. marina is distinguished from the other two by many anatomical characters, notably its possession of a 'tail', denuded of appendages and continually renewed from reserve segments at the base. The other species differ from each other in minor points only. The anatomical characteristics of *marina* can perhaps be regarded as adaptations to its rather special habitat; an explanation along these lines has been attempted in the case of the tail (Wells, 1950).

The behaviour of *marina* has been studied by several investigators; that of the others has received very little attention. In *marina*, the behaviour of the intact worm has been shown to be dominated, to a remarkable extent, by two spontaneously active pacemakers. The evidence on this point is briefly reviewed in an accompanying paper (Wells & Albrecht, 1951). We thought it worth while to make comparative studies of the other species, to see whether differences of behaviour organization would appear, to parallel the known differences in structure and habitat. We were however unsuccessful with *branchialis*,* and the following account is restricted to *ecaudata*, a species which affords good material for this kind of investigation.

The work was done at the Plymouth laboratory of the Marine Biological Association of the United Kingdom, in September 1949 and January 1950.

BRAINLESS ISOLATED EXTROVERT PREPARATIONS

The isolated extrovert preparation consists essentially of the proboscis with a length of the oesophagus attached. It also includes most of the retractor muscle sheath and of the first diaphragm. In *marina*, it exhibits '*f* cycles', i.e. an alternation of vigorous rhythmic contraction and comparative rest, the pacemaker being the oesophageal wall (see preparations M. 36 and M. 37 in Fig. 1).

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* In a series of seventeen brainless isolated extroverts of *branchialis*, set up exactly as described for *ecaudata*, the great majority failed to give satisfactory tracings. The lever strokes were feeble or absent, even in the case of preparations which had been seen to contract vigorously and rhythmically in a bowl of sea water before they were mounted on the apparatus. Only two good records were obtained. One was like a typical *ecaudata* tracing (e.g. E. 30 in Fig. 1). The other—the most vigorous of the series—gave a quite exceptional record, like one obtained from an atypical *marina* oesophagus by Wells (1937, fig. 3, lower half). The available specimens of *branchialis* were rather smaller than those of *ecaudata*; partly for this reason, and partly because of our unsatisfactory experience with the extroverts, we made no attempt to set up more complicated preparations of *branchialis*.

We made a series of thirty isolated extrovert preparations from *ecaudata*, taking care to exclude the brain. They were suspended in sea water, at temperatures ranging from 11·4 to 18·2° C., and with lever weights of 0·12, 0·2 or 0·4 g. In eighteen of the experiments, brainless *marina* extroverts were simultaneously suspended in the same bath, and recorded in exactly the same way, to reduce to a minimum the possibility that the very striking differences observed between the two species were due to the experimental conditions.

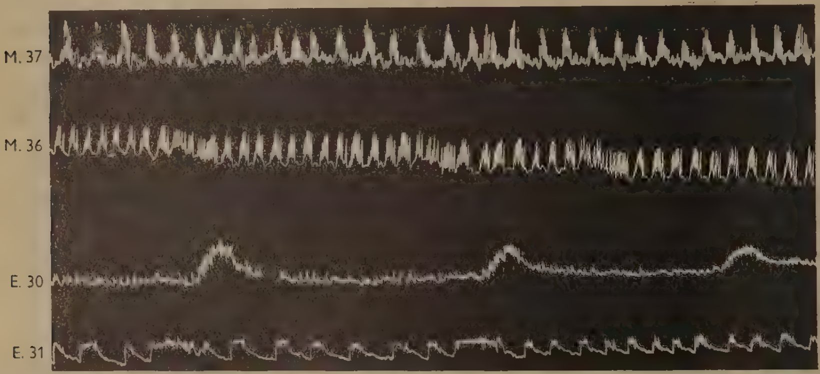

Fig. 1. Four brainless isolated extroverts, the upper two from *A. marina* and the lower two from *A. ecaudata*. Preparations M. 37, E. 30 and E. 31 were recorded simultaneously; the trace of M. 36 was added later. The extracts are 4 hr. long. Read all records from left to right.

The results obtained with *ecaudata* were very consistent, and are typified by the trace of preparation E. 30 in Fig. 1. There is a background of activity, either continuous or grouped into very brief outbursts, so closely crowded as almost to run into each other. Against this background, one sees a series of spells of vigorous activity, of long duration and spaced widely apart. Three of these appear in the published extract from E. 30. The interval between the spells is rather variable, but typically about 30–40 min. In many cases, it is shorter than this at the beginning of the experiment, then lengthens out and settles at the usual value. The lower trace (E. 31) shows a preparation in which the spells are still visible, but the background activity is grouped in a way which resembles the *f* cycle of a *marina* extrovert. This particular preparation was however a unique exception; the normal behaviour of the *ecaudata* extrovert is that shown by E. 30.

If now one examines a series of records from brainless extroverts of *marina*, one can often detect prolonged spells of activity, superposed on the *f* cycle and occurring at longer intervals. Three such spells are visible in the extract from M. 36 in Fig. 1. There seems to be little doubt that all of the tracings in Fig. 1 are modifications of a common scheme. Starting from the typical *ecaudata* pattern of E. 30, and passing through E. 31 and M. 36 to M. 37, one can derive the *f* cycle of the *marina* extrovert by supposing the background activity in *ecaudata* to be increased in vigour, and organized into the characteristic intermittence.

The widely-spaced spells resemble the irrigation-defaecation cycles of *marina* in timing, but we think it unlikely that the two are related. The extrovert spells are generally suppressed, in both species, when the extrovert is connected with the central nervous system, as in the experiments described in the next section. They may perhaps be related to the 'secondary rhythm' reported in the oral half of the *marina* oesophagus by Wells (1937). We have no suggestions to offer about their functional significance.

EXTROVERT BODY-WALL PREPARATIONS

We made a series of experiments on *ecaudata* by the method illustrated in Fig. 1 of the accompanying paper, to find out whether the extrovert pattern of this species has any influence on the behaviour of the whole worm. The gills of *ecaudata* are borne by all segments from the 16th or 17th to the hind end of the body; the irrigation waves, by which water is driven through the burrow, run forwards from the hinder extremity, and die out at about the level of the most anterior gill. In seventeen cases, we recorded from the extrovert and an anterior body-wall strip. In a further ten, we added one or two tracings from the branchiate part of the body. The experiments were performed, some in September 1949, at temperatures from 17·5 to 20·5° C., and some in January 1950, at 10·8–15·5° C. The levers were weighted at 0·2 or 0·4 g.

The behaviour of the extrovert was quite different, in the majority of these experiments, from that shown by the isolated brainless preparations. It now gave a series of vigorous activity bursts, of very variable duration and frequency, and exactly correlated with bursts in the various body-wall lines. An example is given in Fig. 2. The bursts can be clearly seen along the whole length of the body; they are superposed, in the case of the branchiate segments, on a rather regular oscillation that may represent irrigation waves.

On dividing the nerve cord just behind the head, there was definite inhibition of the part of the worm behind the cut in fifteen out of twenty-five cases, and doubtful inhibition in five. This effect is temporary, and is paralleled in the case of *marina*. When it has passed off, the hinder end of *ecaudata* shows vigorous and fluctuating activity patterns, like those of the whole worm before the cut was made; and similar patterns are also visible in the anterior portion (extrovert, nerve ring and brain). As long as some part of the central nervous system is present, the activity patterns of *ecaudata* are more variable than in the case of *marina*, not only from preparation to preparation, but also from time to time in the same preparation.

The pictures given by the two species are in obvious contrast. The extrovert of *marina* traces its characteristic *f* cycle, whether isolated from the central nervous system or not; in the latter case, its pattern is transmitted to the body wall. On the other hand, the behaviour of the *ecaudata* extrovert is usually greatly changed when the brain and nerve cord are present; the extrovert now appears to be dominated by activity patterns of central nervous—or, at least, non-oesophageal—origin. It may not be irrelevant that the brain of *ecaudata* is very much better developed than that of *marina* (Wells, 1950).

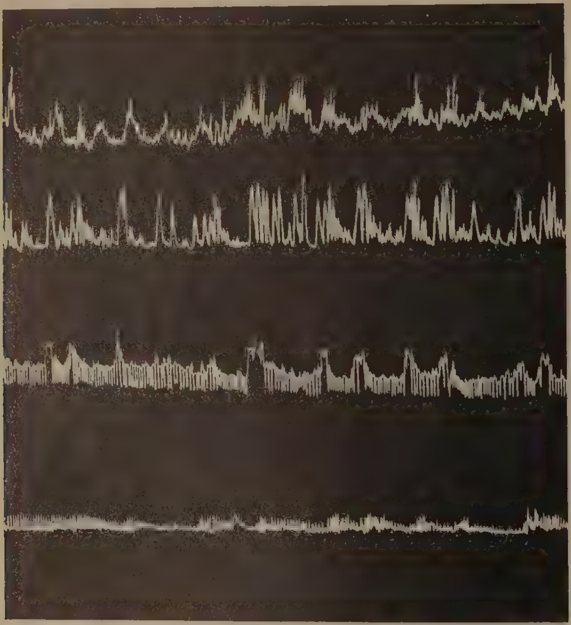

Fig. 2. *A. ecaudata.* Simultaneous recording of the movements of the extrovert (top line), anterior segments (second line), front part of branchiate region (third line) and hind part of branchiate region (lower line). The extract is 2 hr. long, and begins 4 hr. after the start of the experiment. (Worm A.E. 30.)

In five of the twenty-seven experiments in this series, the extrovert gave tracings like that of an isolated, brainless extrovert for at least a large part of the time. In a further four, there were short periods during which the 'brainless' pattern appeared. The remainder, making two-thirds of the whole, gave only the 'central nervous' type of activity described above.

An extract from one of the preparations whose extrovert behaved in the 'brainless' manner is shown in Fig. 3. The major activity spells of the extrovert have no influence on the body wall. Its background activity is however closely correlated with that of the anterior body-wall strip, as one can see by running a set-square along the record. It is tempting to suppose, since we have homologized the background activity of the *ecaudata* extrovert with the *f* cycle of *marina*, that this correlation is due to a flow of oesophageal influences into the central nervous system. Unfortunately, owing to the great variability of the behaviour of the innervated *ecaudata* body wall, even after its connexion with the brain and extrovert has been severed, this suggestion could not be checked with any certainty by the means at our disposal.

The main conclusion is that the extrovert of *ecaudata* is more subordinate than that of *marina*. The organization of the activity of the *marina* extrovert into a regular 6–7 min. intermittence is accompanied by the assumption—or the great intensification—of its pacemaker role. The habitat of *ecaudata* differs from that typical of *marina*, and the mode of life of the former species has not yet been described in any detail. From preliminary observations which we have made, and hope to develop more fully, its habits do not seem to differ very fundamentally from those of many littoral errant polychaetes. The adaptations which have enabled *marina* so successfully to colonize the tidal sand flats are not only anatomical, but include the very distinct and specialized structure of its burrow. Perhaps the organization of its behaviour into cycles of great regularity is part of the same story.

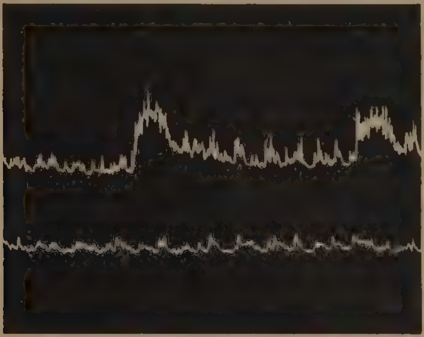

Fig. 3. *A. ecaudata.* Simultaneous recording of the movements of the extrovert (above) and anterior segments (below). The extract is 1½ hr. long and begins 1 hr. 40 min. after the start of the experiment. (Worm A.E. 18.)

SUMMARY

1. *Arenicola ecaudata* differs not only in structure, but in mode of life, from *A. marina.* Our results indicate that there are also great differences in behaviour physiology.

2. The brainless isolated extrovert of *ecaudata* traces a continuous, or nearly continuous, background of activity, upon which prominent spells of vigorous rhythmic contraction appear at intervals of the order of 30–40 min. Similar spells are sometimes shown by the corresponding preparation from *marina*, whose characteristic *f* cycle can be regarded as produced by the organization of the background activity of *ecaudata* into vigorous and regularly spaced outbursts.

3. There is little evidence of a pacemaker role of the oesophagus in *ecaudata*. If the movements of the extrovert and body wall are simultaneously recorded, they generally exhibit correlated outbursts of variable and fluctuating pattern, and very

unlike the behaviour of the brainless extrovert. Similar outbursts are shown by the body wall after severance of its connexion with the extrovert. They are probably of central nervous origin.

REFERENCES

WELLS, G. P. (1937). Studies on the physiology of *Arenicola marina* L. I. The pacemaker role of the oesophagus, and the action of adrenaline and acetylcholine. *J. Exp. Biol.* **14**, 117–57.

WELLS, G. P. (1950). The anatomy of the body wall and appendages in *Arenicola marina* L., *Arenicola claparedii* Levinsen and *Arenicola ecaudata* Johnston. *J. Mar. Biol. Ass. U.K.* **29**, 1–45.

WELLS, G. P. & ALBRECHT, E. B. (1951). The integration of activity cycles in the behaviour of *Arenicola marina* L. *J. Exp. Biol.* **28**, 41–50.

THE FUNCTION OF HAEMOGLOBIN IN RELATION TO FILTER FEEDING IN LEAF-MINING CHIRONOMID LARVAE

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(With One Text-figure)

Tendipes (= *Chironomus*) *plumosus* larvae are unique among species of *Tendipes* in employing a filter-feeding mechanism for obtaining their food (Walshe, 1947*a*). The larva spins a sheet of salivary secretion across the lumen of its tube, after which, by rapid anteroposterior undulations of the body, it irrigates the tube. Plankton or other organic particles in the incoming water current are thereby caught in the salivary net and subsequently eaten. This filter feeding is an energetic process involving glandular activity in spinning the salivary sheet and muscular activity in maintaining a rapid current of water during the filtration of food particles. A study (Walshe, 1950) of the amount of filter feeding at different oxygen concentrations has shown that this is essentially an aerobic process, declining at low oxygen concentrations and absent under anaerobic conditions. Normal larvae are incapable of feeding below about 10% air saturation of the water, while larvae without functional haemoglobin (i.e. after carbon monoxide treatment) cease to feed at 26% air saturation. Even above this value larvae with carboxyhaemoglobin feed less than normal animals. Thus the haemoglobin, in addition to its established significance as an oxygen carrier at low oxygen pressures (Harnisch, 1936; Ewer, 1942) and during recovery from oxygen lack (Harnisch, 1936; Walshe, 1947*b*), also plays a part in enabling the larvae to continue an active aerobic feeding process even when little oxygen is available.

Other chironomid larvae, namely, the leaf-mining genera, employ a filter mechanism for obtaining their phytoplanktonic food and, although the mechanism is slightly different from that of *T. plumosus* in that the larvae turn round in their tubes after spinning the net (Burtt, 1940), the principle is the same and also demands considerable activity on the part of the animal. Some of the species of these genera contain abundant haemoglobin, so a number of experiments was made to see whether in these larvae also the blood pigment is of functional value whilst filter feeding.

Larvae of two filter-feeding genera, namely, *Glyptotendipes* and *Endochironomus*, were removed from the leaves of their host plants and put into straight glass tubes of the same dimensions as their natural burrows in or on the plants. The larvae lived well in these glass tubes, lining them with salivary secretion, filter-feeding normally and remaining in them until the end of pupation. Their feeding behaviour could be clearly seen within these tubes, which were stuck vertically through holes in a horizontal celluloid sheet suspended in an aquarium in which the respiratory conditions of the water could be controlled. In this way several larvae could be observed simultaneously. The amount of filter feeding of normal larvae was compared with that of carbon monoxide-treated larvae at a range of different oxygen concentrations. At each oxygen concentration between five and ten larvae were studied; after an initial 2 hr. under the desired respiratory conditions the numbers of larvae filter feeding were determined at half-hourly intervals for the next 3 hr. Larvae filter feeding could be distinguished from those irrigating the tube for respiratory purposes or carrying on other activities, by the very characteristic net-spinning movements and by the nets, made visible by phytoplankton added to the experimental water. Experiments were carried out in a dilute phytoplankton suspension, since larvae failing to catch any suspended matter in their nets soon abandon filter feeding. The larvae were observed in a dim light in order to lessen any change in oxygen concentration due to photosynthesis by the plankton. The experiments were made at room temperature (14·5–18° C.).

The first leaf-mining larva studied was *Glyptotendipes pallens* Meig., a red or dark red larva containing much haemoglobin (according to Scheer (1934) the concentration is twice that in the *Tendipes plumosus* group), and collected from *Stratiotes* and *Potomogeton* in a pond. The amount of filter feeding at different oxygen concentrations, with and without functional haemoglobin, is given in Table 1 and Fig. 1 A. In well-aerated water the carbon monoxide-treated larvae fed as much as normal animals, but at lower oxygen concentrations they always showed less filter feeding than those with functional haemoglobin. The same result was obtained with *Endochironomus dispar* Meig., another red chironomid found mining in dead *Phragmites* stalks (Fig. 1 B). In these two species therefore the haemoglobin plays the same role in filter feeding as it does in *Tendipes plumosus*.

Other species of *Endochironomus*, however, have considerably less haemoglobin in their blood, *E. tendens* Fabr. being pink rather than red in colour and *E. albipennis* Meig. being very pale pink or colourless. These two species, incidentally, were found in habitats where the average oxygen content of the water was probably considerably higher than that in the stagnant peaty pond from which *E. dispar* was collected. From the previous experiments it was expected that these larvae would be less efficient filter feeders at low oxygen pressures than the red larvae, since they had less pigment to pick up oxygen with which to supply their demands. Such, however, was not the case (Table 1, Fig. 1 C, D). They were capable of maintaining their filter feeding at reduced oxygen concentrations as effectively as their haemoglobin-containing relatives. Carbon monoxide treatment caused a slight diminution in amount of feeding in the pink species (*E. tendens*) and had no effect in the very pale species (*E. albipennis*). These carbon monoxide experiments incidentally serve as a useful check on the validity of drawing conclusions about the function of haemoglobin from carbon monoxide treatment. Such conclusions are always based on the assumption that the carbon monoxide only eliminates the oxygen-carrying capacity of the haemoglobin and has no adverse effect on other physiological processes. Here its action of diminishing the filter feeding of chironomids containing haemoglobin must be due solely to its effect on the pigment, since it has no effect on larvae without haemoglobin.

Presumably therefore, these two pale species have a capacity for maintaining an output of energy under partly anaerobic conditions of a different nature from that of the red species. To see whether the resistance to total anaerobiosis threw any light on their behaviour at low oxygen concentrations, ten larvae of each of the four species were kept in nitrogenated water in large bottles, and their rate of death during the course of a number of days was recorded. Under such conditions none of the larvae filter fed, but for a number of hours they maintained a continuous irrigation current through their tubes, after which they left their tubes, became increasingly immobile and finally died. Of the four species, *Glyptotendipes* and *Endochironomus dispar* remained in their tubes and were active longest, a few individuals surviving for 89 hr., whereas the pale *Endochironomus* species were all out of their tubes and immobile or dead considerably before this. Thus the haemoglobin-containing species have the greater capacity for complete anaerobiosis, although in surroundings totally

Table 1. *The amount of filter feeding of four species of leaf-mining chironomid larvae at different oxygen concentrations, with and without carboxyhaemoglobin*

Species	Normal animals			Animals with carboxyhaemoglobin		
	% air saturation	No. of animals	% observations in which larvae filter feeding	% air saturation	No. of animals	% observations in which larvae filter feeding
Glyptotendipes pallens Meig.	100–80	8	58	100–80	9	67
	36	8	62	38	6	43
	15	9	53	19	6	37
	13	9	60	15	9	24
	8	9	28	7	8	0
	<3	6	0	<3	5	0
Endochironomus dispar Meig.	100–80	5	67	100–80	4	63
	50	8	70	54	7	60
	39	7	63	41	6	37
	24	7	54	25	7	37
	18	5	44	19	4	0
	7	5	0	9	4	0
Endochironomus tendens Fabr.	100–80	11	91	100–80	10	80
	15	6	93	19	7	91
	13	3	87	15	5	68
	8	4	19	7	3	0
	<3	6	0	<3	5	0
Endochironomus albipennis Meig.	100–80	5	90	100–80	5	85
	50	7	83	54	7	88
	39	8	75	41	7	80
	24	5	72	25	5	64
	18	4	30	19	5	20
	7	4	0	9	5	0

devoid of oxygen this can have no direct connexion with the possession of the blood pigment.

Because of their inhibitory action on certain oxygen-utilizing cellular enzymes, respiratory poisons may also be used to explore animals' powers of anaerobic activity. The resistance of *Glyptotendipes pallens* and *Endochironomus tendens* to potassium cyanide was therefore tested. Larvae in glass tubes were put into M/5000- and M/10,000-KCN solutions in aerated pond water. At both concentrations the larvae abandoned filter feeding and behaved as if under anaerobic conditions, ultimately coming out of their tubes. After 24 hr. all larvae of both species recovered when removed from the cyanide. There was thus no striking difference in total susceptibility of the two species at these concentrations. The effects of greater concentrations were not explored, since even in the stronger of these solutions the unnatural twitchings of the larvae indicated that the cyanide was having a physiological effect other than the desired one of blocking part of the respiratory process. At both concentrations, however, there was a difference between the species in the rate at which the signs of respiratory poisoning appeared (as indicated by their abandoning respiratory irrigation and leaving the tubes), the pale *Endochironomus* larvae remaining in their tubes longer than the red *Glyptotendipes*. This would seem to

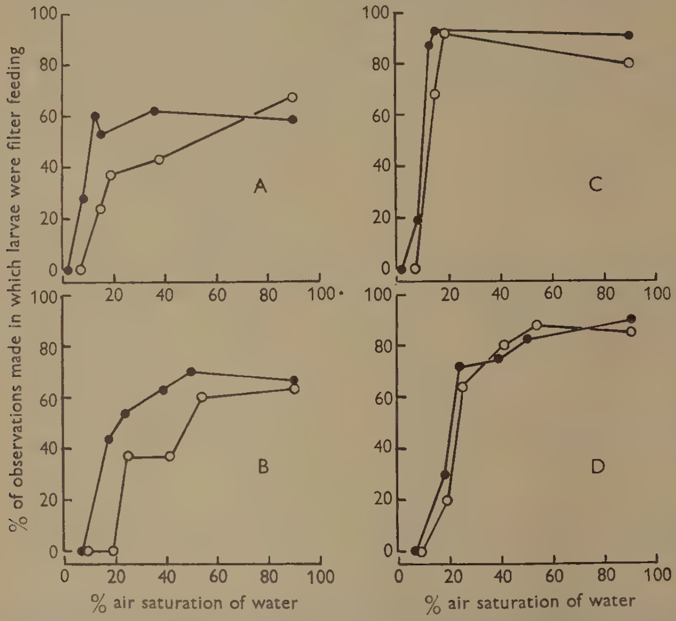

Fig. 1. The relation between oxygen concentration and filter feeding of chironomid larvae with and without carboxyhaemoglobin. ● Normal larvae, ○ larvae with carboxyhaemoglobin. A, *Glyptotendipes pallens*; B, *Endochironomus dispar*; C, *Endochironomus tendens*; D, *Endochironomus albipennis*. Data from Table 1.

indicate that the pale species is less immediately inconvenienced by an inability to utilize oxygen through the oxidase-dehydrogenase system. The capacity of the pale larvae to filter feed in the presence of little oxygen may thus be related to the possession of an alternative system of respiratory enzymes efficient at low oxygen concentrations, at which the red species, on the other hand, need haemoglobin as an oxygen source. This suggestion must be a tentative one, however, since it is based only on a difference in speed of susceptibility of the two species as judged by relatively few data.

However, the fact remains that the haemoglobin of red leaf-mining chironomids is of functional value to them in carrying on filter feeding at low oxygen pressures, as it is in *Tendipes plumosus*. Nevertheless, the possession of haemoglobin is not the only respiratory modification in the chironomids for activity under partly anaerobic conditions, since other larvae can maintain active feeding without the intervention of the blood pigment.

SUMMARY

1. The filter feeding of four species of leaf-mining chironomid larvae with and without functional haemoglobin was studied at different oxygen concentrations.

2. Two red species, *Glyptotendipes pallens* and *Endochironomus dispar*, showed at low oxygen concentrations a reduced amount of filter feeding after treatment with carbon monoxide (to render the haemoglobin functionless). The blood pigment is therefore of significance to these larvae in increasing the amount of feeding possible at low oxygen concentrations.

3. Two pale species, *Endochironomus tendens* and *E. albipennis*, however, despite their having little haemoglobin, were nevertheless capable of filter feeding in water poor in oxygen.

4. The capacity to live anaerobically was greatest in the red species.

5. In potassium cyanide solutions, on the other hand, the red *Glyptotendipes pallens* larvae were more rapidly affected than the pale *Endochironomus tendens* larvae. It is tentatively suggested, therefore, that the ability of the larvae poor in haemoglobin to filter feed at low oxygen concentrations may be due to their possession of respiratory enzyme systems, alternative to the cyanide-sensitive type, which are efficient at low oxygen pressures.

My grateful thanks are due to Prof. Kaj Berg in whose laboratory in Hillerød, Denmark, this work was done.

REFERENCES

Burtt, E. T. (1940). A filter-feeding mechanism in a larva of the Chironomidae (Diptera: Nematocera). *Proc. R. ent. Soc. Lond.* A, **15**, 113.

Ewer, R. F. (1942). The function of haemoglobin in *Chironomus*. *J. exp. Biol.* **18**, 197.

Harnisch, O. (1936). Primäre und sekundäre Oxybiose der Larve von *Chironomus thummi* (nebst ergänzenden Messungen an *Tubifex tubifex*). *Z. vergl. Physiol.* **23**, 391.

Scheer, D. (1934). Die Farbstoffe der Chironomidenlarven. *Arch. Hydrobiol.* **27**, 359.

Walshe, B. M. (1947*a*). Feeding mechanism of *Chironomus* larvae. *Nature, Lond.*, **160**, 474.

Walshe, B. M. (1947*b*). The function of haemoglobin in *Chironomus* after oxygen lack. *J. Exp. Biol.* **24**, 329.

Walshe, B. M. (1950). The function of haemoglobin in *Chironomus* under natural conditions. *J. Exp. Biol.* **27**, 73.

OSMOTIC REGULATION IN MOSQUITO LARVAE: THE ROLE OF THE MALPIGHIAN TUBULES

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(*Received* 3 *July* 1950)

(With Three Text-figures)

INTRODUCTION

In a recent paper (Ramsay, 1950*a*) an investigation of the osmotic relations of the larvae of *Aedes* species was reported. Measurements of freezing-point depression were made on fluids collected from various parts of the body. In the case of *A. aegypti*, a fresh-water species, it was found that the fluid eliminated from the anus was strongly hypotonic to the haemolymph; the fluid entering the rectum from the intestine—presumably originating in the Malpighian tubules—was isotonic with the haemolymph. It was therefore concluded that the osmotic work required to produce a hyptonic 'urine' was carried out by the rectal epithelium and that the Malpighian tubules made no significant contribution to osmotic work. There is no reason to doubt this conclusion; but it is not to be taken to imply that the fluid excreted by the tubules is identical with the haemolymph in composition. The total concentration of the various osmotically active substances is virtually the same in both fluids, but their individual concentrations may be widely different. Evidence that the Malpighian tubules of *Limnophilus* larvae are concerned in ionic regulation has been presented by Boné & Koch (1942), who showed that the concentration of chloride in the intestinal fluid can be very different from that of the haemolymph.

It has not proved possible to collect intestinal fluid from a mosquito larva in quantity sufficient for the determination of chloride. But a method has recently been worked out whereby it is possible to determine sodium in small volumes of fluid, of the order of 10^{-3} cu.mm., and this has afforded a means of attacking the problem. The investigation now to be described is in the nature of an extension of the earlier work, and, to anticipate, it is demonstrated that although the fluid excreted by the Malpighian tubules has virtually the same osmotic pressure as the haemolymph, its sodium concentration can be very much lower.

MATERIAL AND METHODS

Larvae of *Aedes aegypti* were bred as before from eggs and were fed on a preparation of powdered dog biscuit. In the third instar they were removed to clean media and thereafter starved. The media used were distilled water, 0·75 and 1% NaCl. The distilled water was changed twice per week. The larvae were allowed to remain in the same medium for at least 5 days before being used for experiment.

Certain improvements were made in the method of collecting fluid from the intestine. In the earlier work the larva was secured by two ligatures, one around the neck and the other around the respiratory siphon. Although there was good reason to believe that obstruction of the tracheal system was without effect on osmotic regulation in *Aedes* larvae, this feature of the technique was felt to be undesirable. It proved possible to construct a fine wire snare by which the base of the respiratory siphon could be held firmly but without constriction. With the larva secured in this way the spiracles were allowed to break the surface and could be observed to open and close in a natural manner.

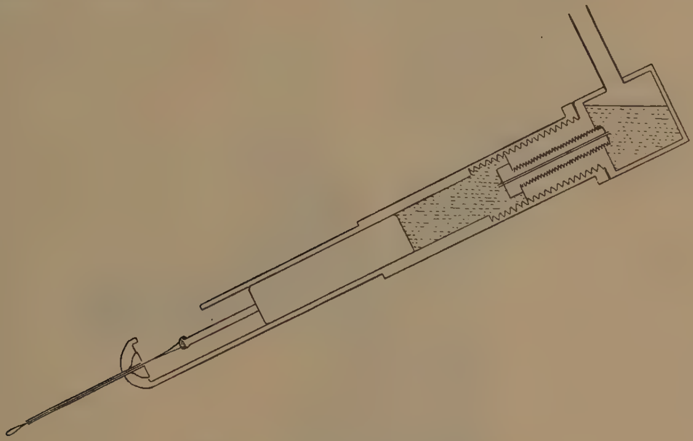

Fig. 1. For explanation see text.

The snare is illustrated in Fig. 1. It consists of a loop of tungsten wire, 0·002 in. thick, passed through a hypodermic needle. The ends of the loop are attached to a piston working in a cylinder which is filled with water and communicates with a second chamber through a fine glass capillary. A rubber tube connected to the second chamber is held in the operator's mouth. By blowing or sucking, the operator can extend or retract the loop, and the resistance offered by the capillary retards the movements of the piston so that accurate control is possible.

The neck of the larva was tightly ligatured as before. A glass cannula of about 100 μ diameter, mounted upon a micromanipulator, was inserted through the anus and rectum into the intestine as described in the earlier paper, and served to collect the droplets of fluid passing down the intestine. The larva, thus secured and cannulated, is illustrated in Fig. 2. It was also found possible, by stretching the larva, to straighten out the loop in the intestine sufficiently to allow the cannula to be passed right up to the pyloric chamber if necessary. Haemolymph was collected at the end of the experiment by drying the larva and puncturing it on a slide under liquid paraffin.

Although this technique is satisfactory in that the tracheal system can function normally and the anal gills are in free communication with the haemolymph, the disadvantage still remains that the midgut is open to the intestine and the fluid collected is not necessarily derived from the Malpighian tubules alone. The forward bend taken by the Malpighian tubules after their origin from the pyloric chamber makes it impossible to close off the midgut by a ligature applied to the outside of

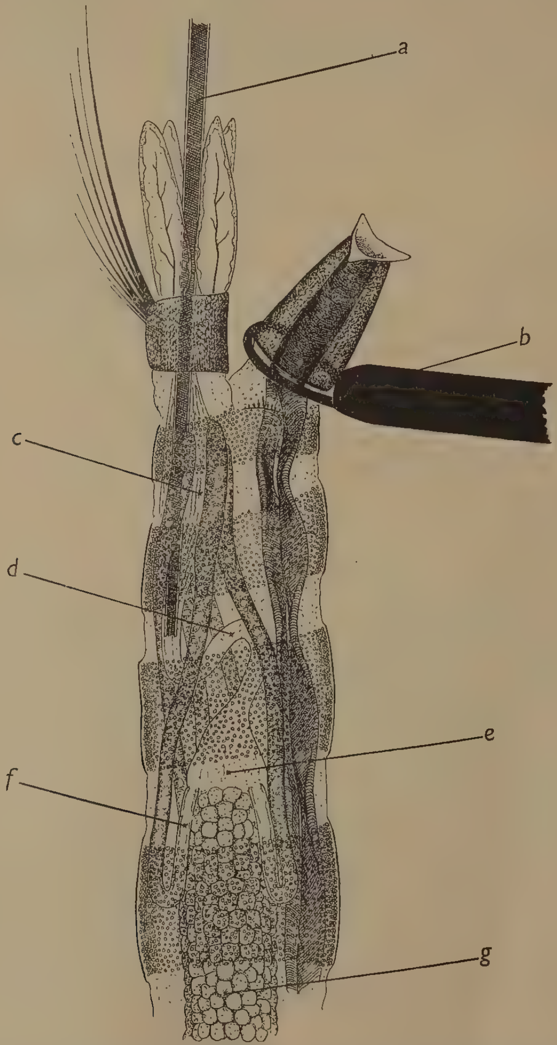

Fig. 2. Collection of intestinal fluid. *a*, cannula filled with liquid paraffin (stained with Sudan III) and inserted into posterior part of intestine; *b*, snare holding base of respiratory siphon; *c*, rectum with distal regions of Malpighian tubules applied to it; *d*, loop of intestine; *e*, pyloric chamber; *f*, proximal region of Malpighian tubule; *g*, midgut.

the larva. Attempts to obstruct the midgut just anterior to the pyloric chamber with droplets of liquid paraffin or bubbles of air were unsuccessful. Some experiments were therefore carried out on preparations of the Malpighian tubules removed from the animal, in which circumstances isolation of the midgut was possible.

The larva was placed on a slide under liquid paraffin. The body was first cut through between thorax and abdomen and then torn apart just anterior to the respiratory siphon. In this way the gut and Malpighian tubules were drawn out of the remains of the body. A fine silk ligature was then tied around the midgut just anterior to the pyloric chamber, and a cannula was inserted into the pyloric chamber via the intestine. In such a preparation the tracheal system was of course destroyed, and the question arose whether a sufficient supply of oxygen reached the tubules where they lay in a drop of haemolymph under a thin layer of liquid paraffin. It was not possible to make any direct measurement of the oxygen tension in the haemolymph, but an indication was obtained by adding a little ox blood to the preparation; spectroscopic examination showed that the haemoglobin remained well oxygenated for an indefinite period. This point having been established, the addition of ox blood was of course avoided in experiments in which fluids were taken for analysis.

Collections were also made directly from the Malpighian tubules removed from the body either (*a*) distally, from a point about one-quarter of the way down the tubule from its blind end, or (*b*) proximally, from a point just before the opening of the tubule into the pyloric chamber. The technique was very much the same as that employed on the earthworm nephridium (Ramsay, 1949*b*). The operation was carried out on a slide to which a cover-glass had been fixed to provide a 'step', and the tubule was kept in a drop of haemolymph under liquid paraffin. In (*a*) a fine silk ligature was tied around the tubule at the appropriate distance from the blind end, and the knot was held against the 'step' by a needle (in this case, of tungsten) while the collecting pipette was inserted. After the insertion a small droplet of liquid paraffin was extruded from the pipette to seal off the rest of the tubule from the puncture, and the point of the pipette was then thrust farther in so as to lie in the lumen beyond the droplet (see Fig. 3). In (*b*) no ligature was applied. The intestine was held down over the 'step' by the needle, the pipette was thrust through the wall of the pyloric chamber and worked into the tubule through the natural opening; the droplet of liquid paraffin was then extruded as before. These operations were carried out under the high-power binocular. The quantities of fluid collected were $2\text{–}3 \times 10^{-3}$ cu.mm.

After collection the samples of intestinal fluid and haemolymph were transferred to watch-glasses varnished internally with Bakelite 'Damarda' lacquer and filled with liquid paraffin; the lacquer, being hydrofuge, prevents the watery droplets from spreading over the surface of the watch-glass. The much smaller samples taken directly from the Malpighian tubules were retained in the collecting pipette and transferred directly to other pipettes. Measurements of osmotic pressure were carried out by the freezing-point method (Ramsay, 1949*a*) and measurements of sodium by an adaptation of flame photometry (Ramsay, 1950*b*; Ramsay, Falloon & Machin, 1951). The freezing-point measurements were carried out in duplicate and the sodium measurements in triplicate. Both osmotic pressure and sodium concentration are expressed in terms of the equivalent concentration of NaCl per cent. The figures for osmotic pressure (based on duplicates) are believed to have a probable error of $< \pm 0.01\%$ NaCl, and those for sodium (based on triplicates) to have a probable error of $< \pm 0.02\%$ NaCl. The '*t*' test for significance has been used, and the values of '*P*' are taken from Fisher & Yates (1938). $P < 0.05$ is considered to be significant and $P < 0.01$ to be highly significant. Table 1 gives the protocol of a single experiment.

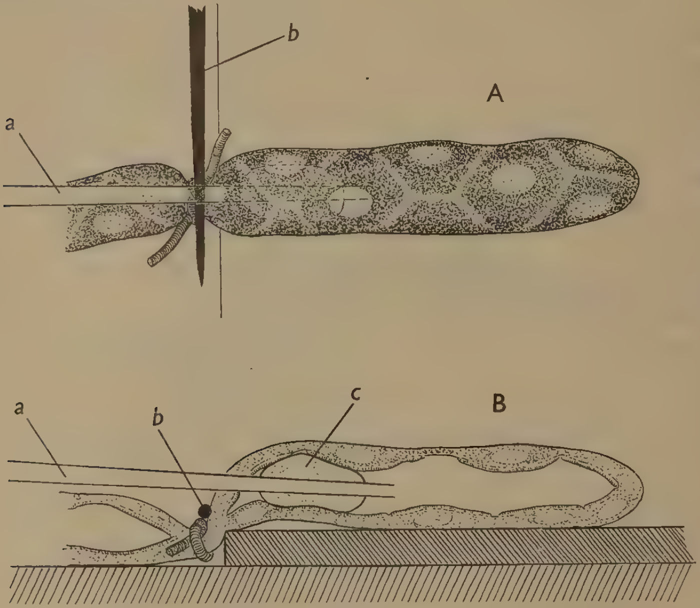

Fig. 3. Collection of fluid from distal region of Malpighian tubule. A, from above, as seen by operator; B, as imagined in vertical section. *a*, collecting pipette; *b*, tungsten needle; *c*, droplet of liquid paraffin.

RESULTS

Part I. Work was begun on the collection of intestinal fluid from larvae reared in distilled water and the results are presented in Table 2. The osmotic pressure of the intestinal fluid is seen to be a little lower than that of the haemolymph; the sodium concentration is very much lower, the difference being 0·37% NaCl, nearly 60% of the sodium concentration of the haemolymph.

It therefore appears that, although the power of the tubule to set up differences in osmotic pressure is negligible, a substantial difference in the concentration of sodium can be brought about. In reaching this conclusion, however, the assumption is made that the fluid collected from the intestine originates in the Malpighian tubules; the possibility that the concentration difference is brought about by the cells of the midgut cannot wholly be excluded. In order to put this to the test collections were made from the intestine in preparations having the midgut isolated by a ligature as

Table 1. *Serial 7*

Flame photometer

		Deflexion			Deflexion
1.	0·5 % NaCl	192	7.	Intestinal fluid	99
2.	0·5 % NaCl	189	8.	Haemolymph	248
3.	0·5 % NaCl	198	9.	Intestinal fluid	100
4.	Haemolymph	246	10.	0·5 % NaCl	188
5.	Intestinal fluid	96	11.	0·5 % NaCl	190
6.	Haemolymph	247	12.	0·5 % NaCl	190

	Average deflexion	Concentration	
		Calibration curve	% NaCl
0·5 % NaCl	191	12·5	0·50
Haemolymph	247	17·5	0·70
Intestinal fluid	98	5·85	0·23

Freezing-point

(Distilled water: freezing-point, 2·990° C.; 1·00 % NaCl≡ Δ0·600° C.)

	Freezing-point	Δ° C.	% NaCl	
Haemolymph	2·550	0·440	0·900	av. 0·908
Haemolymph	2·540	0·450	0·916	
Intestinal fluid	2·540	0·450	0·916	av. 0·916
Intestinal fluid	2·540	0·450	0·916	

	Osmotic pressure (% NaCl)	Sodium concentration (% NaCl)
Haemolymph	0·91	0·70
Intestinal fluid	0·92	0·23

Table 2. *Larvae from distilled water*

Serial	Osmotic pressure as % NaCl				Na as % NaCl			
	Haemo-lymph	Intestinal fluid	Diff.		Haemo-lymph	Intestinal fluid	Diff.	
			+	−			+	−
1	0·83	0·82		0·01	0·52	0·25		0·27
2	0·85	0·81		0·04	0·65	0·37		0·28
3	0·75	0·63		0·12	0·65	0·31		0·34
4	0·68	0·60		0·08	0·65	0·18		0·47
5	0·72	0·65		0·07	0·69	0·25		0·44
6	0·70	0·65		0·05	0·62	0·22		0·40
			Av. −0·065 *P* <0·01				Av. −0·37 *P* <0·01	

described in the preceding section. The results of these experiments are given in Table 3, and it can be seen that they are in conformity with the results given in

Table 2. We are therefore at liberty to believe that differences in sodium concentration between intestinal fluid and haemolymph are attributable to the activity of the Malpighian tubules.

The Malpighian tubules therefore play an active part in the retention of sodium in the haemolymph of larvae which are reared in distilled water; it now remains to discover whether their activity can be adapted to assist in getting rid of sodium under conditions in which this ion tends to accumulate in the haemolymph. The work of Boné and Koch on *Limnophilus* suggests that in this animal the fluid excreted by the tubules has a lower chloride concentration than the haemolymph when the external medium is poor in chloride, and a higher concentration when the external medium is rich in chloride. It would be of interest to discover a comparable relationship in the sodium balance of *Aedes*.

Table 3. *Larvae from distilled water* (*preparation*)

Serial	Osmotic pressure as % NaCl				Na as % NaCl			
	Haemolymph	Intestinal fluid	Diff.		Haemolymph	Intestinal fluid	Diff.	
			+	−			+	−
7	0·91	0·92	0·01		0·70	0·23		0·47
8	0·82	0·85	0·03		0·75	0·18		0·57
9	1·13	1·05		0·08	0·78	0·38		0·40
10	0·72	0·65		0·07	0·51	0·27		0·24
			Av. −0·027 P=0·05–0·04				Av. −0·42 P < 0·01	

Table 4. *Larvae from* 0·75 % NaCl

Serial	Osmotic pressure as % NaCl				Na as % NaCl			
	Haemolymph	Intestinal fluid	Diff.		Haemolymph	Intestinal fluid	Diff.	
			+	−			+	−
11	0·95	0·90		0·05	0·77	0·80	0·03	
12	0·92	0·83		0·09	0·81	0·65		0·16
13	1·14	1·01		0·13	1·10	0·69		0·41
14	0·96	0·91		0·05	0·85	0·60		0·25
15	0·98	0·90		0·08	0·94	0·59		0·35
16	0·95	0·92		0·03	0·98	0·38		0·60
			Av. −0·072 P < 0·01				Av. −0·29 P=0·05–0·01	

Experiments were next carried out on larvae adapted to 0·75 % NaCl. The results of these experiments, given in Table 4, show, first, that the sodium concentration of the haemolymph is greater than in larvae adapted to distilled water and, secondly, that the sodium concentration of the intestinal fluid is still significantly lower than that of the haemolymph. But the difference in sodium concentration is now smaller, both in the absolute sense (0·29 % NaCl as compared with 0·37 % NaCl) and in the relative sense (32 % of the sodium concentration of the haemolymph as compared with 59 %).

Table 5. *Larvae from* 1% NaCl

Serial	Osmotic pressure as % NaCl				Na as % NaCl			
	Haemolymph	Intestinal fluid	Diff.		Haemolymph	Intestinal fluid	Diff.	
			+	−			+	−
17	1·15	1·23	0·08		1·33	1·36	0·03	
18	1·16	1·12		0·04	1·15	1·14		0·01
19	1·54	1·50		0·04	1·07	1·02		0·05
20	1·41	1·26		0·15	1·36	1·39	0·03	
21	1·33	1·20		0·13	1·31	1·19		0·12
22	1·13	1·08		0·05	1·11	1·08		0·03
			Av. − 0·05 $P = 0·2–0·1$				Av. − 0·02 $P = 0·4–0·3$	

Further to test this point the experiments were repeated on larvae adapted to 1% NaCl. This external concentration is definitely harmful as judged by increased death-rate, but the larvae taken for use were active and appeared healthy. Table 5 gives the results of this series of experiments, and it can be seen that the sodium concentration of the intestinal fluid now closely approaches that of the haemolymph but certainly does not exceed it. It may therefore be said that the sodium-retaining activity of the tubule is capable of adaptation in respect of the availability of this ion, but not to the extent of an actual reversal of direction as is the case with chloride in *Limnophilus*.

It will be observed that in certain cases, e.g. serials 16, 17, 18, 20, the sodium concentration (as % NaCl) is greater than the osmotic pressure (as % NaCl) of the same fluid. There is, of course, no anomaly in this; it may mean either that some of the sodium is not in free solution, or, more probably, that sodium ions are in electrical balance with polyvalent anions.

Part II. In the vertebrate kidney it has long been known that the primary process of urine formation is the separation of an ultrafiltrate of the plasma in Bowman's capsule, and that the composition of this ultrafiltrate is modified during its subsequent passage through the tubule. Evidence has accumulated to show that an analogous process occurs in the excretory organs of many invertebrates (Picken, 1936, 1937; Krogh, 1938). The analogy is less easy to trace in the case of the Malpighian tubules of insects. In cryptonephric insects, such as the mealworm, it has been suggested that a process of ultrafiltration occurs in the modified distal portions of the tubules which are applied to the rectum (Poll, 1934; Patton & Craig, 1939). On the other hand, in their more usual arrangement the Malpighian tubules have been compared with the aglomerular nephrons of certain fishes (Boné & Koch). Even if there is no primary process of ultrafiltration it does not necessarily follow that urine of constant composition is uniformly excreted over the whole length of the tubule. Wigglesworth (1931) has described two regions in the Malpighian tubules of *Rhodnius*, of different histological appearance and with different physiological properties. In the distal region of the tubule the cells contain granules and the striated border next to the lumen is of the 'wabensaum' type; in the proximal region there are no granules and the striated border is of the 'börstensaum' type. Fluid is secreted into the lumen in the distal region and resorbed in the proximal region, and there is evidence that both these processes may be selective in respect of dissolved substances.

In *Rhodnius* the two regions of the tubule are sharply discontinuous; in *Aedes* there is a gradual transition in histological appearance around the bend of the forwardly directed loop. In the distal region, which is of slightly greater diameter, the tubule appears opaque owing to the presence of granules; in the proximal region, just before the opening into the pyloric chamber, the cytoplasm becomes clear and transparent. Pagast (1936) has shown that the density of the granules (vacuoles, in his account) is related to the external medium in which the larvae are kept, the density being greatest in distilled water and least in solutions of NaCl. The presence of these granules makes it very difficult to study the striated border. In the course of the present work an examination was made of the tubules of larvae reared in different media. The tubules were examined fresh, in droplets of haemolymph under liquid paraffin, and were compared with tubules of *Rhodnius* similarly mounted. In the *Aedes* tubule the striated border at the proximal end is easily seen; it is usually sharply demarcated from the lumen by a clear edge, and while in some tubules there may be a slight fraying of this edge there is no unmistakable 'börstensaum' as in *Rhodnius*. Over the distal region the striated border is more difficult to observe, but it appears to be demarcated from the lumen by a sharp and slightly scalloped edge, characteristic of 'wabensaum'. The striated border is on the whole thinner in *Aedes* than in *Rhodnius*, but this may be related to the apparently greater distension of the tubules in *Aedes*. Wigglesworth (personal communication) examined the tubules of *Aedes* but was not able to recognize two distinct regions as in *Rhodnius* nor to decide that the tubule of *Aedes* could be identified with one or other of the two regions of the tubule of *Rhodnius*. Other descriptions of the Malpighian tubules of mosquitoes (Roubaud, 1923; Missiroli, 1925, 1927; de Boissezon, 1930) are mostly incomplete and generally based on fixed material. Lecaillon (1899) described 'prolongements ciliformes' of the living cells of the midgut and Malpighian tubules in *Culex pipiens*, but this observation does not seem to have been confirmed. A difference in function between the two ends of the tubule in *Aedes* could hardly be argued on the histological evidence.

In the present attempt to obtain physiological evidence larvae adapted to distilled water were used. The collections of fluid from distal and proximal regions were made as already described and were analysed for sodium only. The results are presented in Table 6. As far as the proximal region of the tubule is concerned little comment is required; there is a clear difference in sodium concentration between tubule fluid and haemolymph, of the same order as that given in Table 2. The fluid collected from the distal region shows a similar difference, but it is smaller and the variance is much greater. In one case, serial 25, the tubule fluid is identical with the haemolymph.

It is not particularly difficult to thrust the pipette into the lumen of the tubule, even at the distal end, but once inside the point is almost completely obscured by the granules. If penetration has been successful, the droplet of liquid paraffin can be seen to occupy the lumen of the tubule as it is extruded from the pipette. But it is then necessary to thrust the pipette farther in, so that its point lies beyond the drop-

Table 6. *Larvae from distilled water. Collection from Malpighian tubules direct*

Serial	Na as % NaCl							
	Distal region				Proximal region			
	Haemolymph	Tubule fluid	Diff.		Haemolymph	Tubule fluid	Diff.	
			+	−			+	−
23	0·39	0·22		0·17	—	—		—
24	0·38	0·29		0·09	—	—		—
25	0·37	0·37		(0·00)	—	—		—
26	—	—		—	0·40	0·13		0·27
27	—	—		—	0·44	0·15		0·29
28	—	—		—	0·54	0·11		0·43
29	0·40	0·10		0·30	—	—		—
30	0·48	0·14		0·34	—	—		—
31	0·45	0·17		0·28	—	—		—
32	0·51	0·25		0·26	—	—		—
33	—	—		—	0·46	0·15		0·31
34	—	—		—	0·49	0·22		0·27
35	—	—		—	0·48	0·12		0·36
			Av.	−0·24			Av.	−0·32
					$P=0{\cdot}2\text{–}0{\cdot}1$			

let, and this movement has to be made 'blind'. Further, the walls of the Malpighian tubule do not seem to have the same self-sealing properties as are found in the annelid nephridium—which is one reason for sealing with a droplet of liquid paraffin behind the point of the pipette. It is therefore possible that the second thrust of the pipette may tear the wall of the tubule and allow the haemolymph to enter; it is even possible that the point of the pipette may pass right out of the tubule by penetrating the wall on the under side without being observed by the operator. This is very probably what happened in serial 25, and the smaller differences of serials 23 and 24 may possibly be the result of damage to the walls and some entry of haemolymph into the lumen.

In analysing these results serial 25 has been disregarded. The mean difference between tubule fluid and haemolymph is 0·24% NaCl for the distal region and 0·32% NaCl for the proximal region, and the difference between these two figures is not statistically significant.

This result is somewhat unsatisfactory. But if the variance associated with the distal region of the tubule is really due to faulty operative technique as has been supposed, and if one sees no reasonable prospect of eliminating such faults, then it is pointless to accumulate further data. One can only conclude that the experiments have failed to demonstrate a difference in sodium concentration between the two ends of the tubule; but one may add that if such a difference does exist it is unlikely to be greater than 0·1% NaCl.

DISCUSSION

This investigation was undertaken primarily to find the answer to one simple question. It had been shown earlier that the intestinal fluid (presumed to be derived from the Malpighian tubules) was isotonic with the haemolymph. Can the tubule separate a fluid containing more or less sodium than the haemolymph, or is it non-selective as Patton & Craig concluded in the case of the tubules of the mealworm? The answer to this question is that the tubule can separate a fluid containing less sodium.

Arising out of this part of the investigation there are two matters which should not pass unremarked. First, it has been shown that if the midgut is ligatured off the composition of the intestinal fluid is not significantly altered (compare Tables 2 and 3). This might mean that the midgut and the tubules produce fluids of identical composition which then pass down the intestine; but it is very much more likely to mean that the normal flow of intestinal fluid is derived almost exclusively from the tubules—as has often been supposed, though upon very little evidence. Secondly, in the earlier paper the intestinal fluid was stated to be isotonic with the haemolymph; to be precise, the intestinal fluid was found to be hypotonic to the extent of 0·04% NaCl, but this difference was not statistically significant. In the present work the intestinal fluid has been found to be hypotonic to the extent of 0·065% NaCl, and the difference is statistically highly significant. 0·065% NaCl corresponds to less than 10% of the osmotic pressure of the haemolymph, and whatever it may be statistically, this small difference in osmotic pressure does not appear to be of any great physiological significance.

The scope of the investigation was then enlarged to embrace an extension of the original problem. If the tubule is capable of separating a fluid containing less sodium than the haemolymph, is this activity capable of modification under different physiological conditions? The answer to this question is that under conditions in which the sodium concentration of the haemolymph is high the sodium concentration of the tubule fluid approximates to that of the haemolymph; but it does not exceed that of the haemolymph as found by Boné & Koch for chloride in *Limnophilus*. From the figures published by Boné & Koch it might be objected that the relation which they found between the chloride concentration of the 'urine' and that of the external medium might largely be accounted for if the animal swallowed the external medium in considerable quantity and passed it through the midgut to the intestine. In a personal communication Prof. Koch informs me that in the course of the work on *Limnophilus* a certain number of experiments were made in which a ligature was applied to the neck of the larva, and this did not appear to reduce the 'urine' flow; it is therefore likely that the objection is met and that the relation is a real one. In the present state of knowledge we have no reason to suppose that the conclusions of Boné & Koch on *Limnophilus* are incompatible with the conclusions reached in the present work on *Aedes*. Nor is it impossible that the Malpighian tubules of *Corethra* eliminate a hypertonic fluid as is supposed by Schaller (1949); but it is to be noted that this contention is not supported by any analysis of the fluid.

Lastly, an approach was made towards a new and wider problem—the localization of function in Malpighian tubules. The results of this investigation on the tubules of *Aedes* were negative; but at the same time there was very little reason to have expected otherwise. The Malpighian tubules of *Aedes* are short and relatively simple; and when one considers the variety of form presented by the tubules of other insects, not only in themselves but in their relations with other organs of the body, one cannot but feel that if an investigation of this wider problem is to make useful progress very careful consideration ought first to be given to the choice of material. It does not seem likely that such choice would fall upon mosquito larvae.

SUMMARY

1. The part played by the Malpighian tubules in the salt and water balance of *Aedes aegypti* larvae has been studied; the intestinal fluid and haemolymph have been compared in respect of freezing-point depression and sodium concentration.

2. It appears highly probable that the fluid passing down the intestine is derived from the Malpighian tubules with little or no contribution from the midgut.

3. When the larvae are kept in fresh water the intestinal fluid is very slightly hypotonic to the haemolymph (not isotonic as previously reported), but its sodium concentration is only about one-half that of the haemolymph.

4. When the larvae are kept in solutions of NaCl the difference in sodium concentration between intestinal fluid and haemolymph decreases. In an external medium of 1% NaCl the difference is abolished.

5. There is thus evidence that when the external medium is poor in salts the Malpighian tubules can contribute to the work of salt retention by excreting a fluid containing less sodium than the haemolymph; but there is no evidence that under any conditions they can excrete a fluid containing more sodium than the haemolymph.

6. Evidence of a decrease in the sodium concentration of the tubule fluid from distal region to proximal region is not statistically significant.

Once again it is a pleasure to thank Dr Wigglesworth for reading the typescript of this paper and for much valuable discussion.

REFERENCES

Boné, G. & Koch, H. J. (1942). *Ann. Soc. roy. zool. Belg.* **73**, 73.
de Boissezon, P. (1930). *Arch Zool. exp. gén.* **70**, 281.
Fisher, R. A. & Yates, F. (1938). *Statistical Tables.* Oliver and Boyd.
Krogh, A. (1938). *Osmotic Regulation in Aquatic Animals.* Cambridge University Press.
Lecaillon, A. (1899). *Bull. Soc. ent. Fr.* p. 353.
Missiroli, A. (1925). *Riv. Malariol.* **6**, 1.
Missiroli, A. (1927). *Ann. Igiene*, **35**, 113.
Pagast, F. (1936). *Zool. Jb.*, 3. Abt., 56, 184.
Patton, R. R. & Craig, R. (1939). *J. Exp. Zool.* **53**, 437.
Picken, L. E. R. (1936). *J. Exp. Biol.* **13**, 309.
Picken, L. E. R. (1937). *J. Exp. Biol.* **14**, 20.
Poll, M. (1934). *Ann. Soc. roy. zool. Belg.* **65**, 87.
Ramsay, J. A. (1949*a*). *J. Exp. Biol.* **26**, 57.
Ramsay, J. A. (1949*b*). *J. Exp. Biol.* **26**, 65.
Ramsay, J. A. (1950*a*). *J. Exp. Biol.* **27**, 145.
Ramsay, J. A. (1950*b*). *J. Exp. Biol.* **27**, 407.
Ramsay, J. A., Falloon, S. W. H. W. & Machin, K. E. (1951). *J. Sci. Instrum.* (in the Press).
Roubaud, E. (1923). *Ann. Inst. Pasteur*, **37**, 627.
Schaller, F. (1949). *Z. vergl. Physiol.* **31**, 684.
Wigglesworth, V. B. (1931). *J. Exp. Biol.* **8**, 428.

FERMENTATION IN THE RUMEN OF THE SHEEP

I. THE PRODUCTION OF VOLATILE FATTY ACIDS AND METHANE DURING THE FERMENTATION OF WHEATEN HAY AND LUCERNE HAY *IN VITRO* BY MICRO-ORGANISMS FROM THE RUMEN

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It is now generally accepted that the fermentation of carbohydrates in the rumen gives rise to the formation of large amounts of the lower volatile fatty acids; but the fact that these acids are absorbed in considerable amounts and at different rates from the rumen into the blood stream has made it clear that *in vitro* studies of the fermentation processes are necessary in order to ascertain the actual quantities in which they are made available to the ruminant.

The course of carbohydrate dissimilation by the mixed flora and fauna of the rumen is determined primarily by an extraordinarily stable *milieu* (cf. Marston, 1939, 1948), and so it might be expected that a closely similar environment, and inoculation of the substrate with the natural mixed culture of organisms from the rumen, would be essential prerequisites for the fermentation to proceed *in vitro* as it does in the rumen.

The study of pure or of 'purified' cultures of organisms derived from the rumen may eventually illuminate the intermediate stages in the course of the dissimilations which occur there and the nature of the organisms responsible, but it cannot give precise information concerning products of the fermentation of natural fodders in the rumen. The same may also be said of studies made with related organisms derived from other sources—soil, faeces, etc.—or with fermentations carried out at temperatures or reactions which are outside the limits that obtain within the rumen. Most of the experiments described in the earlier literature on the *in vitro* fermentation of refractory carbohydrates—cellulose, etc.—must be included in the above categories.

If information cogent to the nutritional physiology of the ruminant is to be obtained, it is essential that some major characteristics of the fermentation as it proceeds in the rumen should be used as criteria by which to gauge the similarity of the courses taken by *in vitro* and *in vivo* fermentations of natural foodstuffs. The extent to which certain insoluble carbohydrate fractions of natural fodders are broken down in the rumen, and the amount of methane produced from them, can be measured. If these criteria were met *in vitro*, under the appropriate conditions of temperature, reaction and anaerobiosis, then the fermentation might reasonably be considered to have taken a course closely similar to that occurring in the rumen, and the fatty acids produced could be taken to represent those actually formed in the rumen.

EXPERIMENTAL

Samples of wheaten hay and lucerne hay were inoculated with rumen fluid and allowed to ferment *in vitro*. The breakdown of cellulose and hemicellulose and the production of fatty acids were measured; in some of the fermentations the amounts of methane and carbon dioxide formed were determined.

Technique

(1) Samples of the fodders were inoculated with rumen fluid in Erlenmeyer flasks fitted with inlet and outlet tubes. The air was displaced, either before or after the addition of inoculum, by the passage of carbon dioxide or of nitrogen. The anaerobic state was preserved by closing the outlet tube with a water seal. The flasks were incubated at 38–40° C.

(2) In those experiments where the fermentation gases were measured the fodders were placed in a wide-mouthed bottle in a constant temperature water-bath (40° C.). After addition of the inoculum the air above the liquid was displaced by the passage of nitrogen. The gases formed during the fermentation were passed through a drier of 'Dehydrite' and the CO_2 removed by passage through soda lime or 'Sofnolite' guarded by a further drier of Dehydrite. The remaining gas was passed over copper oxide in a furnace (720° C.) for combustion of the methane to carbon dioxide and water. These gases were collected and weighed in absorption tubes. A continuous passage ($\doteq$ 30 l./24 hr.) of CO_2-free air was maintained through an inlet tube opening into the system immediately after the fermentation bottle.

(3) *The inocula* consisted of rumen fluid drawn from sheep with permanent rumen fistulae. A glass tube, with one end covered by a wrapping of surgical gauze over a layer of wire gauze, was inserted into the rumen. A syringe attached to the other end was used to withdraw the sample. Where small inocula were used, tap water was added to the flask, but with large inocula no other liquid was added.

(4) Either calcium carbonate or ammonium carbonate was added with the inoculum to act as buffer during the fermentations.

(5) *Analysis of substrates*. The residual solids from the fermentations were collected, dried at 100° C., and together with samples of the original fodder, were subjected to analysis. Cellulose was determined by the method of Norman & Jenkins (1933); it will be described subsequently as 'N. J. cellulose'. To reduce the number of operations, the method was modified slightly by taking less material for analysis and by increasing the strength of the hypochlorite solutions. For the determination of furfuraldehyde-yielding substances the phloroglucide method, as described by the Association of Official Agricultural Chemists (1940), was used; the furfuraldehyde yield of the N.J. cellulose was also determined so that the group could be separated into what have been called for the purpose of this paper, 'total pentosans', 'free pentosans', and 'cellulose pentosans'.*

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* The fact that these substances have, for convenience, been called pentosans is not meant to imply any exact knowledge of their nature. The group of furfuraldehyde-yielding substances present in such plant tissues is a wide one: it includes pentosans, polyuronides and other substances not so well defined. In general, losses of furfuraldehyde-yielding substances have been taken as an approximate measure of the fermentation of the whole hemicellulose group.

(6) *Analysis of fatty acids formed.* At the end of the fermentation period, after the residual solids had been removed, a suitable aliquot of the liquor was taken for analysis of the mixture of volatile fatty acids present. Analyses were also made of the fatty acids present in the rumen fluid used as inocula to allow for the appropriate corrections to be made in the calculations of fatty acids formed during the fermentation. The liquor was made just alkaline to phenolphthalein and reduced to a small volume. It was transferred to a small flask, 5 g. of magnesium sulphate ($MgSO_4 . 7H_2O$) were added, and the fatty acids liberated by the addition of phosphoric acid, the reaction being adjusted to pH 3–4 (Congo Red). The volume was made up to 7·5 ml. and, by distillation at constant volume, 50 ml. of distillate containing the pure acids was collected. (In some experiments a double distillation was resorted to, and in such cases formic acid was destroyed in the second distillation by the addition of mercuric oxide to the flask.) The recovery of volatile fatty acids in such a distillation was previously found to be: formic acid 92%, acetic acid 98–99%, propionic and butyric acids 100%. In some of the earlier experiments the acids present in this distillate were determined by the procedure of Gray (1947*a*). In the majority of experiments a partition chromatographic method was used. This method is described in detail in the Appendix.

Experiments

Series 1: *small inocula.* The fermentations of wheaten hay were set up in Erlenmeyer flasks, as described above. Calcium carbonate was included as buffer, and a small inoculum of rumen fluid was used to start the fermentation. The period of fermentation was varied from 16 to 120 hr. The data and results of analyses are summarized in Table 1.

Table 1. *Fermentation* in vitro: *series* 1, *small inocula*

Exp. no.	Substrate	Weight of substrate taken (g.)	Inoculum (ml.)	Duration (hr.)	Various fractions fermented (%)*				Acids formed				
					N.J. cellulose	Total pentosan	'Cellulose pentosan'	'Free pentosan'	Formic (%)	Acetic (%)	Propionic (%)	Butyric (%)	Total ac (mmol./ substrat
1	Wheaten hay	100	50	16	—	—	—	—	0	90	4	6	0·42
2		100	50	24	—	—	—	—	6	85	4	5	0·38
3		100	50	24	—	—	—	—	5	85	9	1	0·48
4		1000	500	24	—	—	—	—	3	94	0	3	0·50
5		100	50	24	2·0	14	7·0	20	0	74	20	6	1·35
6		50	25	24	3·8	16	7·2	26	0	67	27	6	2·15
7		100	50	48	—	—	—	—	1	34	41	24	0·94
8		50	25	48	8·3	19	9·0	30	0	30	56	14	2·50
9		100	50	72	5·0	20	10	30	0	47	42	11	2·06
10		100	50	120	7·9	19	16	23	1	27	56	16	2·27

* N.J. cellulose = cellulose as determined by method of Norman & Jenkins.
Calculated as pentosan: total pentosan – total furfuraldehyde-yielding substances; cellulose pentosan = furfuraldehyde-yielding su
stances present in N.J. cellulose; free pentosan = furfuraldehyde-yielding substances not present in N.J. cellulose.

Series 2: *large inocula.* Two different fodders were used in these experiments—wheaten hay and lucerne hay. The procedure in most cases was similar to that used in series 1, but in those experiments where the production of methane was measured the fermentations were carried out according to the second procedure. The inocula, however, were relatively much larger, and ammonium carbonate was substituted for calcium carbonate as buffer. The period of fermentation was in all cases 48 hr. The details of experimental data and results are set out in Table 2.

Table 2. *Fermentation* in vitro: *series 2, large inocula*

Exp. no.	Substrate*	Fodder sample	Percentage of carbohydrate fraction fermented				Acids formed				Methane (ml./g. substrate)
			N.J. cellulose	Total pentosan	'Cellulose pentosan'	'Free pentosans'	Acetic (%)	Propionic (%)	Butyric (%)	Total acid (mmol./g. substrate)	
11	Wheaten hay	1	44	42	41	43	42	48	10	3·3	—
12		1	41	41	40	41	41	49	10	3·3	—
13		2	39	38	40	36	48	37	15	3·0	—
14		2	41	40	42	38	52	35	13	3·3	—
15		2	38	37	35	39	50	36	14	3·2	—
16		2	40	40	38	42	51	35	14	3·8	—
17		2	35	35	35	36	43	49	8	3·5	16
18		2	34	38	37	39	43	42	15	3·1	15
19		2	39	38	39	37	51	34	15	3·2	16
20		3	40	42	40	44	50	39	11	3·8	—
21		3	40	42	38	47	48	41	11	3·6	—
22		3	41	42	41	42	48	41	11	3·7	—
23		3	36	39	36	42	48	42	10	3·7	14
Mean values							47	40	13	3·4	15
24	Lucerne hay	4	35	50	28	60	59	28	13	3·3	—
25		4	36	52	22	64	60	27	13	3·3	—
26		4	38	57	32	68	59	27	14	3·3	—
27		5	41	47	31	56	60	30	10	3·6	19
28		6	36	56	28	68	58	24	18	4·4	—
29		6	38	55	32	66	59	24	17	4·5	—
30		6	29	51	28	61	57	28	15	4·4	21
Mean values							59	27	14	3·8	20

* The air dry weight of substrate taken was 25 g., and the volume of rumen fluid used as inoculum was 750 ml., in each experiment.

DISCUSSION

The experiments were carried out to investigate the conditions necessary for the reproduction *in vitro* of the rumen fermentation, and to determine from such *in vitro* fermentations the amounts of each of the fatty acids made available to the sheep in its rumen.

It has been pointed out that it is desirable to adopt certain criteria from among facts established for the natural fermentation, in order to judge the success of the attempt to reproduce the process *in vitro*. The criteria which can be employed for this purpose are:

(1) *The extent of digestion of the main carbohydrate fractions in the rumen.* It has been shown that in a mixture of wheat straw and lucerne hay about 40% of the cellulose is broken down in the rumen (Gray, 1947*b*). In the digestion of a meadow hay (Marshall, 1949) about the same proportion of the pentosans was fermented.

(2) *The production of methane.* 10–20 l. of methane are produced per kilogram of fodder fed to a sheep; hydrogen is not present in the fermentation gases.

It will be seen from Table 1 that the series of fermentations in which relatively small inocula were employed did not meet the first of these criteria. Very little cellulose was decomposed even after 5 days, and usually after 2 days the odour of the fermenting mass was foul and not at all characteristic of the rumen fermentation. It will also be seen, however, that an effective fermentation of pentosans took place and that the earliest stage was remarkable for the production of acetic acid almost free from other acids. In the later stages larger amounts of the higher acids, and particularly propionic acid, were formed.

In the rumen itself the fodder meets at once with very large numbers of organisms, and so, after the failure of these first experiments, it was decided to increase the size of inocula. Furthermore, it was noticed that the $CaCO_3$ was an inefficient buffering agent in that the reaction changed to about pH 5 in many of the fermentations; ammonium carbonate, which was found to maintain the reaction between pH 6 and 7, was therefore substituted for it. It is evident from Table 2 that the fermentations carried out under these conditions have met the criteria laid down, and there is therefore good reason to claim that the products are similar to those formed in the rumen itself from the same fodders.

(3) *The fatty acids.* From the mean values of fatty acid production in Table 2 it is calculated that a sheep eating 1 kg. of fodder per day would have available from the rumen fermentation the amounts of acids shown in Table 3.

Table 3

	Acetic acid (g.)	Propionic acid (g.)	Butyric acid (g.)	Total acid (g.)
Wheaten hay	96	101	39	236
Lucerne hay	136	76	48	260

Table 4. *Fatty acids in the rumen fluid and in the* in vitro *fermentations*

Fodder	Fermentation	Acetic (%)	Propionic (%)	Butyric (%)
Wheaten hay	*In vitro*	46	41	13
	rumen fluid*	71	17	12
Lucerne hay	*In vitro*	59	27	14
	rumen fluid*	70	14	16

* Averages from a large number of analyses.

In Table 4 the molecular proportions in which these acids were formed *in vitro* are contrasted with the proportions in which they occur in the rumen fluid. The differences between the two groups imply that the rates of absorption of the individual acids differ considerably. The ratio of acetic to propionic acid formed *in vitro* from wheaten hay is very much less than the ratio of the same two acids in the rumen fluid. It must therefore be concluded that propionic acid is more rapidly absorbed than acetic acid when this fodder is fermented in the rumen. A similar examination of the remaining data in this table suggests that, if the products found *in vitro* are in fact produced in the rumen, then the relative rates of absorption of the acids must fall in the following series:

wheaten hay fermented—propionic > butyric > acetic;
lucerne hay fermented—propionic > acetic = butyric.

A discussion of the earlier literature relating to the rates of absorption of the fatty acids from the rumen, and a report of observations which provide confirmation of the findings from the present series of *in vitro* experiments is given in Part II of these studies (Gray & Pilgrim, 1951).

Accumulation of products at the site of fermentation. In the sheep the fatty acids are removed continuously by absorption through the rumen wall, and failure to reproduce this phenomenon *in vitro* may have some influence on the rate of fermentation, and on the proportions in which the acid products are formed. A recent report on the fermentation of cellulose with and without removal of acid products from the site of fermentation (Louw, Williams & Maynard, 1949) showed that there was a reduction in the rate of fermentation, but no change in the proportions of acids formed when these products were allowed to accumulate in the fermentation vessel. In this regard it may be noted that it was necessary in the present work to continue the fermentation for 48 hr. in order to arrive at the same extent of substrate breakdown that was known to take place *in vivo* in 24 hr.

The gases. In a large number of experiments conducted in metabolism chambers in this laboratory the production of methane from a variety of fodders has been accurately measured, and it may be said that from 10 to 20 l. of methane are produced by the sheep from a kilogram of the fodders used in the present series of fermentations. This volume was produced *in vitro*, within 48 hr.; no hydrogen was formed.

The production of CO_2 cannot readily be measured *in vivo* because of the difficulty of separating the fermentation product from the respiratory gases. The amount formed was measured in some of the *in vitro* experiments of the present series, but the data have not been presented as the subject of gas production will be dealt with in a subsequent communication.

Other products. No attention was paid to other products of the rumen fermentation, such as the non-volatile acids and the various neutral bodies.

Microscopic appearance of the fermentation. Insufficient is known of the bacteria responsible for the fermentation to allow them to be used as criteria in the way that chemical changes have been used, and so no detailed microscopic examination of the mixed population of micro-organisms in these fermentations was undertaken. But the protozoa, whatever part they play in these processes, are characteristic members of the population, and since they do not occur outside the normal rumen it is probable that their continued existence *in vitro* would point to a close reproduction of the conditions obtaining in the rumen. In the first, unsuccessful, series of fermentations the protozoa did not survive but in the second series where relatively large inocula were employed they continued to thrive.

SUMMARY

1. When wheaten hay and lucerne hay were fermented by organisms from the rumen of the sheep it was necessary to employ a large inoculum of rumen fluid in order to reproduce the rumen fermentation *in vitro*. With a small inoculum the fermentation did not conform to the known characteristics of the natural process.

2. Products per kilogram of wheaten hay fermented *in vitro* were: fatty acids 200–250 g.—acetic acid 41 %, propionic acid 43 % and butyric acid 16 % (by weight); methane 15 l.

Products per kilogram of lucerne hay were: fatty acids 250–300 g.—acetic acid 53 %, propionic acid 29 % and butyric acid 18 % (by weight); methane 20 l.

3. The findings support the view that, owing to the more rapid absorption of propionic than of the other acids from the rumen, the proportion of this acid remaining in the rumen fluid is considerably less than the proportion actually formed in the fermentation.

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APPENDIX

The separation of volatile fatty acids on a 'celite' partition chromatogram

The following procedure avoids some of the difficulties associated with Elsden's original method (1946) and is in effect an adaptation from that method and the method of Peterson & Johnson (1948). The difficulties inherent in the preparation of silica gel, and the variable behaviour which it exhibits are avoided by the use of celite. A more important gain, however, is that acetic acid is readily eluted from the column and can therefore be determined directly by titration. If other acids, moving more slowly on the column, are present in the mixture analysed, Elsden's method does not separate them from acetic.

Reagents

1. *Celite* diatomaceous silica analytical filter aid (Johns-Manville). Dried overnight at 105° C. before use.
2. *Moir's indicator solution.* A solution of Moir's (modified methyl orange) indicator, 0·75–1·50 mg./ml. in CO_2-free aqueous solution.
3. *BB*1. Thiophene-free benzene containing 1 % by volume of pure *n*-butanol.
4. *BB*7·5. Benzene with 7·5 % by volume of *n*-butanol. Immediately before use in the preparation or development of columns, these developer solutions are shaken with CO_2-free water and filtered through a pad of cotton-wool.
5. *Standard* N/10 NaOH. Protected from CO_2.
6. *Standard* N/100 NaOH. Protected from CO_2.
7. *Phenol red indicator.* 0·1 % phenol red in 20 % alcoholic solution.
8. *Potassium bisulphate.* Finely powdered, dried overnight at 105° C. before use.

Preparation of solution of acids for analysis

An aliquot sample of aqueous distillate containing 2–3 mmol. of total acid is pipetted into a suitable flask or beaker. Total acid is estimated by adding 1 drop of phenol red solution and titrating to a faint pink end-point with N/10 NaOH.

A further 1 drop of N/10 NaOH is added and the solution evaporated just dry on a water-bath. A minimal amount of water (2–4 drops) is added to redissolve the residue and 3 g. of dry $KHSO_4$ is added. A bent stirring rod is used to break up the mixture to a homogeneous pink powder and to ensure that no drops of moisture remain on the walls of the flask.

The mixture is extracted six or seven times with about 4 ml. of dry BB 7·5 solution, the extracts being filtered through a small pad of glass-wool into a 25 ml. standard flask and made up to volume. Of this solution 1 ml. is subsequently titrated for total acid.

Preparation of the column

The tubes are 35 cm. long, 12 mm. internal diameter, constricted at the bottom to support a glass bead or sintered glass disk, and spun out to a funnel at the top. A cork fits in the top, containing a tube which leads, through a 2-way tap, to a reducing valve on a nitrogen cylinder. A tightly packed pad of glass-wool covers the glass bead to a depth of $\frac{1}{16}$ in., but this is not required when a sintered glass disk is used.

For each column 5 g. of dry celite is mixed thoroughly in a mortar with 3 ml. Moir's indicator solution. A slurry of this is made with wet BB 1, and added to the tube small amounts at a time. Rapid packing may be effected by using a piece of glass tubing, one end of which is covered by silk or linen supported by a circle of metal gauze. The covered end should fit snugly into the chromatograph tube.

Separation of acids

The rate of flow through the column is adjusted to about 1 ml./min., a pressure of 3–5 lb./sq.in. being necessary at the surface.

The level of excess BB 1 is taken down until it just enters the surface of the column. A 1 ml. aliquot of the BB 7·5 solution of the acids being estimated is added, taken down to the surface and washed in with 1 ml. BB 1. A further 10 ml. of BB 1 is added and run through the column. By this means the butyric and propionic acids are moved away from the acetic acid which remains near the top of the column. The effluents are collected in 16 × 4 cm. Pyrex boiling tubes. Collection of the butyric effluent is begun before the butyric band reaches the bottom of the tube, and collection of propionic begun when the propionic band is almost ready to emerge.

The flow may be stopped at any stage by opening the tube to air through a two-way tap.

Development with BB 7·5 is begun when the leading edge of the propionic band reaches within 2 cm. of the bottom of the column and continued throughout the collection of propionic and acetic acids. The elution of acetic acid is continued until 8–10 ml. has been collected in addition to that required to leave no trace of pink on the column.

If the formic acid concentration is sufficiently small, the formic band will stay near the top of the column, which may then be used again. Columns should be left with the surface covered and the lower end sealed.

It is necessary to discard the first analysis made on a newly prepared column.

Titration of acids

Titrations are carried out in an atmosphere of CO_2-free N_2, with vigorous mechanical stirring. The N_2 is bubbled and the mixture agitated for 3 min. before titrating. Standard alkali is run in from a 10 ml. CO_2-protected burette.

N/100 NaOH is standardized against N/100 HCl, using 5 ml. N/100 HCl, 15 ml. CO_2-free water, 15 ml. BB 1 and 2 drops of phenol red.

The total acid in the BB 7·5 solution being analysed is estimated by titrating 1 ml. in the presence of 15 ml. BB 1 and 20 ml. CO_2-free water. The effluents containing the individual acids are titrated after adding 20 ml. of CO_2-free water. Two drops of phenol red indicator are used in all titrations and the end-point, which must be approached slowly, is taken as the first appearance of a definite, orange-free, pink colour.

REFERENCES

ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS (1940). *Official and Tentative Methods of Analysis*, 5th ed. Washington: Association Official Agricultural Chemists.
ELSDEN, S. R. (1946). *Biochem. J.* **40**, 252.
GRAY, F. V. (1947*a*). *J. Exp. Biol.* **24**, 11.
GRAY, F. V. (1947*b*). *J. Exp. Biol.* **24**, 15.
GRAY, F. V. & PILGRIM, A. F. (1951). *J. Exp. Biol.* **28**, 83.
LOUW, J. G., WILLIAMS, H. H. & MAYNARD, L. A. (1949). *Science*, **110**, 478.
MARSHALL, R. A. (1949). *Brit. J. Nutr.* **3**, 1.
MARSTON, H. R. (1939). *Ann. Rev. Biochem.* **8**, 557.
MARSTON, H. R. (1948). *Biochem. J.* **42**, 564.
NORMAN, A. G. & JENKINS, S. H. (1933). *Biochem. J.* **27**, 818.
PETERSON, M. H. & JOHNSON, M. J. (1948). *J. Biol. Chem.* **174**, 775.

FERMENTATION IN THE RUMEN OF THE SHEEP

II. THE PRODUCTION AND ABSORPTION OF VOLATILE FATTY ACIDS DURING THE FERMENTATION OF WHEATEN HAY AND LUCERNE HAY IN THE RUMEN

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(With Two Text-figures)

INTRODUCTION

Although the composition of the mixture of volatile fatty acids in the rumen of the sheep may be determined, it can throw no light on the composition of the mixture actually formed in the fermentation unless the relative rates of absorption of the acids are known, for it has been established that they are readily absorbed through the rumen wall into the blood stream (Barcroft, McAnally & Phillipson, 1944). Some attempts have been made (Barcroft *et al.* 1944; Danielli, Hitchcock, Marshall & Phillipson, 1945; Gray, 1947, 1948) to determine these relative rates of absorption, but it seems certain that the results must remain of doubtful significance in view of the fact that the experimental conditions under which they were obtained were too far removed from the normal physiological state of the animal. In fact, while Gray (1947) concluded that propionic acid was more rapidly absorbed than the other acids, the English School, whose work is summarized by Elsden & Phillipson (1948) arrived at the opposite conclusion, namely that acetic acid is more rapidly absorbed than either propionic or butyric acid. The only practicable approach to the problem of the amounts of the fatty acids actually formed in the rumen is by an examination of the fermentation *in vitro*. The results of experiments in this field have been reported in Part I of the present studies, and it is clear that the findings imply certain relative rates of absorption of the acids *in vivo*. It is important that these rates should be checked, if possible, by evidence derived from the animal in its normal state.

It is known that during the digestion of the natural fodders in the rumen there may be a considerable increase in the concentration of fatty acid there immediately after feeding, followed later by a decrease to the original level. It might be expected that if fatty acids are produced in the proportions found *in vitro*, proportions very different from those in the rumen fluid, then the composition of the rumen fluid should undergo certain changes during the fermentation. In the period of an increasing rate of production of acid these changes should reflect the difference in composition between the mixture formed and the mixture previously present in the rumen fluid. During the period of decreasing rate of production of acid, however, the changes should reflect the effects of the different rates of absorption of the individual acids. In short, if the rate of production of acid is great enough to bring about a temporary, but marked, increase in the concentration of acid in the rumen, then the composition of the acid there should change towards the composition of the mixture being formed, and later should gradually revert to the composition of the original rumen fluid. In the experiments described here, such changes in the composition of the rumen fluid have been observed, and it is considered that they provide strong evidence in support of the findings reported in Part I of these studies.

EXPERIMENTAL

Sheep in which permanent rumen fistulae had been established were fed a constant ration for a period of 7 days. On the last day samples of rumen fluid were withdrawn at intervals after feeding, and analyses of the fatty acids were made by the procedure, including the chromatographic method, described in Part I of this work (Gray, Pilgrim & Weller, 1951).

Series 1. One of two sheep was fed on wheaten hay and the other on lucerne hay. On the day before sampling the fodder was removed at 5 p.m. and on the day of sampling it was offered at 10 a.m. and again removed at 5 p.m.

Series 2 was a repetition of series 1 except that fodder was offered at 10 a.m. and removed at 5 p.m. throughout the whole week, and more frequent samples of rumen fluid were taken. The results of the analyses are given in Tables 1 and 2.

Table 1. *Volatile fatty acids present in the rumen fluid, series 1*

Fodder	Time after feeding (hr.)	Fatty acids mmol./100 ml. rumen fluid				Percentages of individual acids		
		Acetic	Propionic	Butyric	Total	Acetic	Propionic	Butyric
Wheaten hay:	0	3·40	0·82	0·68	4·90	69·4	16·7	13·9
	½	3·91	1·14	0·76	5·81	67·3	19·6	13·1
	1	4·46	1·38	0·92	6·76	66·0	20·4	13·6
	4	5·72	2·28	1·34	9·34	61·3	24·4	14·3
	7	6·68	2·54	1·58	10·8	61·9	23·5	14·6
	12	7·28	2·14	1·48	10·9	66·8	19·6	13·6
	24	5·70	1·40	1·15	8·25	69·1	17·0	13·9
Lucerne hay:	0	3·58	0·76	0·80	5·14	69·6	14·8	15·6
	½	7·75	2·08	1·34	11·2	69·4	18·7	11·9
	1	9·38	2·78	1·61	13·8	68·1	20·2	11·7
	4	12·3	3·32	1·94	17·6	70·1	18·9	11·0
	7	13·8	3·61	1·88	19·3	71·5	18·7	9·8
	12	13·4	3·30	1·97	18·7	71·8	17·7	10·5
	24	5·17	1·22	1·18	7·57	68·3	16·1	15·6

DISCUSSION

The data in Tables 1 and 2 show that the rate of production of fatty acid was sufficient to bring about a considerable increase in the concentration of acid in the rumen after feeding. By the end of the day the concentration had returned more or

Table 2. *Volatile fatty acids present in the rumen fluid, series 2*

Fodder	Time after feeding (hr.)	Fatty acids mmol./100 ml. rumen fluid				Percentages of individual acids		
		Acetic	Propionic	Butyric	Total	Acetic	Propionic	Butyric
Wheaten hay:	0	5·87	1·58	1·24	8·69	67·6	18·1	14·3
	½	6·04	1·89	1·43	9·36	64·5	20·2	15·3
	1	6·95	2·50	1·77	11·2	61·9	22·3	15·8
	2	8·39	3·54	2·19	14·1	59·4	25·1	15·5
	3	8·48	3·67	2·22	14·4	59·1	25·5	15·4
	4	10·6	4·76	2·84	18·2	58·2	26·2	15·6
	6	12·1	5·48	2·98	20·5	58·8	26·7	14·5
	8	8·26	3·52	1·86	13·6	60·5	25·8	13·7
	12	9·64	3·57	1·97	15·2	63·5	23·5	13·0
	16	8·82	2·76	1·62	13·2	66·8	20·9	12·3
	20	7·76	2·13	1·53	11·4	67·9	18·7	13·4
	24	6·28	1·49	1·22	8·99	69·8	16·6	13·6
Lucerne hay:	0	6·48	1·38	1·43	9·29	69·8	14·8	15·4
	½	8·93	2·13	1·48	12·5	71·2	17·0	11·8
	1	11·2	2·83	1·80	15·8	70·7	17·9	11·4
	2	14·6	4·08	2·28	21·0	69·7	19·4	10·9
	3	17·4	4·78	3·03	25·2	69·0	19·0	12·0
	4	17·6	4·93	3·00	25·5	68·9	19·3	11·8
	6	15·0	4·10	2·45	21·6	69·7	19·0	11·3
	8	15·9	4·15	2·28	22·3	71·2	18·6	10·2
	12	16·6	3·95	2·23	22·8	72·9	17·3	9·8
	16	13·3	2·91	2·04	18·3	73·0	15·9	11·1
	20	9·56	2·22	1·75	13·5	70·5	16·5	13·0
	24	6·90	1·65	1·42	9·97	69·3	16·5	14·2

less to its original value. It is clear that quite appreciable changes occurred in the composition of the mixture of acids during the day.

It has been pointed out that, to be consistent with the production in the rumen of a mixture of acids similar in composition to that already found *in vitro* (Part I), these changes should proceed in a certain direction when the rate of production of acid is increasing, and in the reverse direction when the production declines. It is not possible to know precisely when the rate of production of acid reached its peak in these experiments, because the concentration of acid may have continued to rise for a considerable time after the rate of production began to decline. However, in the sheep which were fed on wheaten hay it may be said that the rate of production began to decrease within 12 hr. after feeding in series 1, and within 6 hr. in series 2. When lucerne hay was fed the maximum rate of production must have occurred earlier than in the case of wheaten hay.

The changes in the composition of the rumen fluid are reviewed more clearly in the graphs drawn in Figs. 1 and 2, where the ratio of each pair of acids is considered separately. As an example, changes in the ratio [acetic acid]/[propionic acid] when wheaten hay was fed, may be considered (Fig. 1). It can be seen that in the period when the rate of production of acid was increasing this ratio decreased in value—a change consistent with the production of a mixture of acids containing a smaller proportion of acetic acid and a larger proportion of propionic acid than was originally present in the rumen fluid. Furthermore, in the latter part of the day (12–24 hr. after feeding) when the production rate was low and the effects of absorption predominated, the ratio [acetic acid]/[propionic acid] increased to approximately its original level. It is a reasonable inference that this change was due to the more rapid absorption of propionic than acetic acid.

Any other interpretation of these changes would seem to involve the postulation of very considerable alterations in the relative rates of production and/or absorption of the fatty acids during the fermentation and, while there is no *a priori* reason why this should not be so, the interpretation given not only accounts directly for the changes, but is also consistent with the findings which have been discussed in the *in vitro* studies of the rumen fermentation (Part I). There it was predicted that changes very similar to those which have been observed should take place in the composition of the rumen fluid after the feeding of these fodders. The remaining data, examined and interpreted in the same way, suggest that when wheaten hay was fermented the

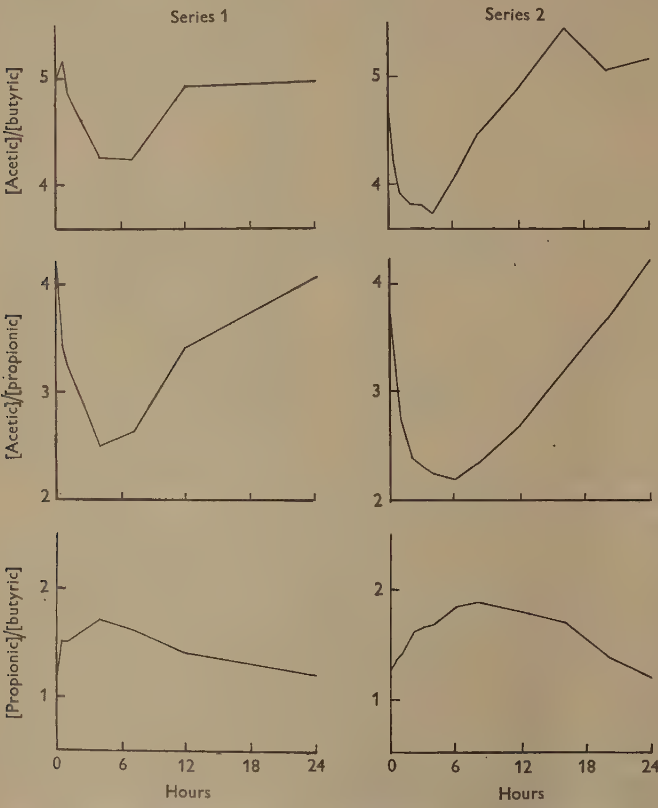

Fig. 1. The ratios [acetic]/[butyric], [acetic]/[propionic] and [propionic]/[butyric] in the rumen during the fermentation of wheaten hay.

relative rates of absorption of the acids fell in the series propionic acid > butyric acid > acetic acid, and that in the case of lucerne hay the order was propionic acid > acetic acid > butyric acid. In view of these conclusions it is necessary to make a critical survey of the earlier work in the field.

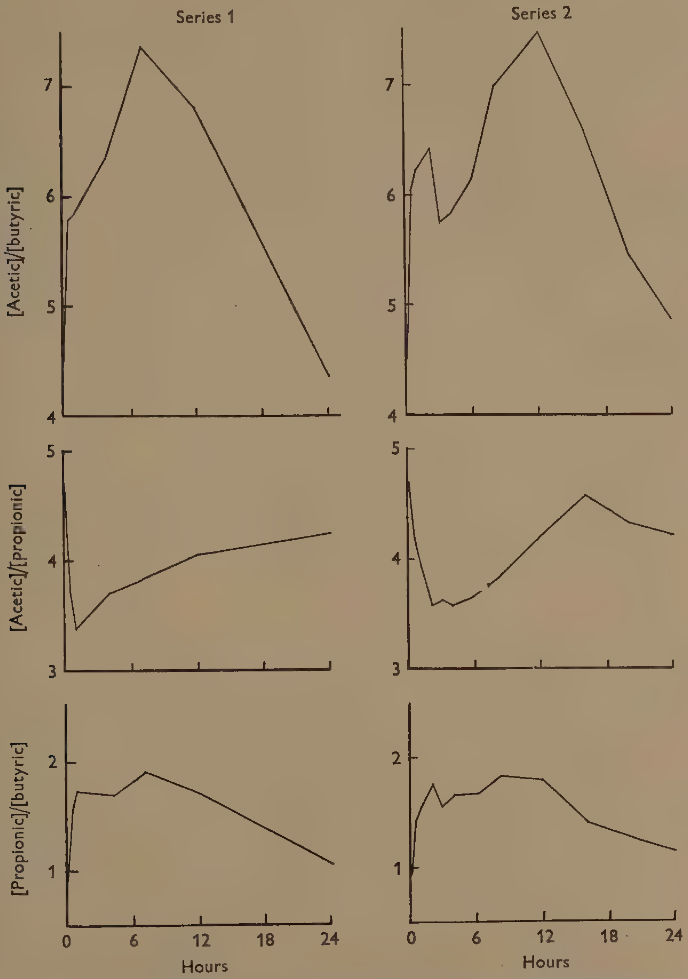

Fig. 2. The ratios [acetic]/[butyric], [acetic]/[propionic] and [propionic]/[butyric] in the rumen during the fermentation of lucerne hay.

The absorption of fatty acids from the rumen was first studied in England by Barcroft and his colleagues (Barcroft *et al.* 1944) who approached the problem from two different aspects. First, they compared the proportions of the individual acids present in the fatty acid of the rumen fluid with those in the fatty acid of the venous blood draining the rumen wall. Because the latter contained the greater proportion of acetic acid they concluded that acetic acid was absorbed more rapidly than were the higher acids. Secondly, they introduced solutions of the sodium salts of each of the three acids (undoubtedly at a reaction higher than pH 7) into an isolated, empty rumen, and measured the concentration of volatile acid developed in the rumen venous blood in each case. They found the concentration to be highest after the introduction of sodium acetate and lowest after the introduction of sodium butyrate. The results then, appear to confirm their first finding.

The work of the English School was continued (Danielli *et al.* 1945) by examining further the absorption of pure acids or their salts when introduced into the empty rumen. The three fatty acids were introduced simultaneously and the mixture remaining in the rumen was analysed at intervals. In one group of experiments the reaction was maintained at pH 6·5, and under these conditions it was established that the relative rates of absorption fell in the series butyric > propionic > acetic. In the other group of experiments the reaction was maintained at pH 7·5, and it was claimed that this led to a reversal of the relative rates of absorption, acetic > propionic > butyric. It has been pointed out, however (Gray, 1948), that the data indicated rather that under these alkaline conditions the rates of absorption were equal.

Meanwhile, work in this laboratory (Gray, 1947, 1948) had covered some of the same ground. From the loss of acids in mixtures introduced into the rumen at pH 6·5 it was found that propionic acid was absorbed more rapidly than acetic acid, but the only experiment in which butyric acid was included suggested that the relative rates of absorption of the three acids fell in the series propionic > butyric > acetic. Furthermore, it was claimed that under alkaline conditions no absorption took place.*

In discussing this earlier work it is necessary to bear in mind that a most important factor in the normal physiological state of the rumen is the reaction of its contents.

The early investigations in this subject, in which material from slaughtered animals was used, may be disregarded. The examination *in vitro* of material taken from living animals has yielded more consistent results. Thus Hale, Duncan & Huffman (1932) found an average reaction of pH 6·8 for animals fed on lucerne hay and Kick, Gerlaugh, Schalk & Silver (1938) gave a range of pH 5·5–7·7 also for lucerne hay; Phillipson (1942) found a range of pH 5·7–6·7 for a sheep fed a mixture of oats, bran, hay and straw. The most reliable values, however, must be obtained by measurements made within the rumen itself. Smith (1941), using a glass electrode *in vivo*, found averages of less than pH 6·27 and 6·0 for cattle fed on lucerne, and on beet pulp plus lucerne, respectively. Gray (1946), also using a glass electrode *in vivo* found a range of pH 5·5–6·8 for sheep fed on lucerne hay, the lowest values occurring soon after feeding. When the animal is starved the reaction rises to pH 7 or higher after the first 24 hr. (Phillipson, 1942; Gray, 1946).

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* This latter claim has since been found only to be correct in certain circumstances. Under alkaline conditions absorption takes place to a varying degree, as found by Danielli *et al.* (1945). A number of factors have been shown to influence the extent of absorption when the rumen contents are alkaline (Jarrett, Gray & Rodda, 1949).

It may be concluded that the reaction of the normal rumen is decidedly acid and that, therefore, any experimental work carried out with the rumen contents alkaline can have no direct bearing on the problem of absorption from the rumen. Thus one part of the evidence from Barcroft *et al.* (1944) in favour of the more rapid absorption of acetic acid than of the higher acids is disposed of.

The evidence relating to the fatty acids in the blood may be criticized on two grounds. In the first place no account was taken of the acids present in the arterial blood supplying the rumen wall. The experimental data included analyses of blood from the carotid artery which showed acid concentrations ranging from 0 to 13 ml. N/100 volatile acid/100 ml. blood, while the rumen venous blood showed a range of 17–75 ml. N/100 volatile acid. The actual proportions of the individual acids in each case were not stated and so, even if the methods of analysis were sound, it is not possible to decide that their final conclusion was justified.

A more important criticism concerns these methods of analysis. It has already been pointed out (Gray, 1947) that the interpretation given to the results of the distillation procedure could not be justified. But the analyses were also checked by the chromatographic method of Elsden (1946). In this method, however, acetic acid is determined as the difference between the total acid and the sum of the propionic and butyric acids, because acetic acid is not eluted from the column.

Now it has been found (Gray, Rodda & Weller, 1949) that the mixture of 'volatile acids' derived from blood by the methods used by Barcroft *et al.* (1944) contains a considerable proportion of acid which is not acetic, propionic or butyric acid. Moreover, this extra fraction consists of at least three acids which all move more slowly down the column than does acetic acid. It is possible then that they were included in the 'acetic' fraction in the work under discussion. To approach the problem of absorption of fatty acids by examination of the fatty acids of the blood would therefore require methods for the separation of the acids from blood which avoided these difficulties. It is clear that the evidence for the absorption of acetic acid at a greater rate than the other acids cannot at present be considered sound.

On the other hand, the work of Danielli *et al.* (1945) suggested that at the reaction of the rumen contents the rates of absorption of the acids should be in the order of butyric > propionic > acetic. The work has been checked in this Laboratory and there is no doubt that, under the particular experimental conditions, the acids are absorbed at these relative rates. But the conditions differ greatly from the normal conditions which obtain in the rumen.

The approach to the problem which has been adopted in the present work avoids the difficulties associated with the animal being in an unphysiological state. Furthermore, the findings are strongly supported by the results of the *in vitro* fermentations reported in Part I of these studies. The difference in the order of the relative rates of absorption of the acids from the two fodders used in these experiments points to the necessity for investigation of the relationship between the concentrations of the acids in the rumen and their rates of absorption from it.

SUMMARY

1. Analyses of the rumen fluid of sheep fed on wheaten hay and on lucerne hay showed that characteristic changes take place in the composition of the mixture of volatile fatty acids in the rumen throughout the day.

2. The changes conform closely to those predicted from the composition of the mixture of fatty acids produced from the same two fodders *in vitro*. They support the view that propionic acid is relatively more rapidly absorbed than either acetic or butyric acid, and that the fermentation of these fodders in the rumen produces a mixture of the acids in which propionic acid forms a larger proportion than it does in the rumen fluid.

The authors again wish to thank the Chief of the Divison, Mr H. R. Marston, F.R.S., for his continued interest and advice throughout this work.

REFERENCES

Barcroft, J., McAnally, R. A. & Phillipson, A. T. (1944). *J. Exp. Biol.* **20**, 120.
Danielli, J. F., Hitchcock, M. W. S., Marshall, R. A. & Phillipson, A. T. (1945). *J. Exp. Biol.* **22**, 75.
Elsden, S. R. (1946). *Biochem. J.* **40**, 252.
Elsden, S. R. & Phillipson, A. T. (1948). *Ann. Rev. Biochem.* **17**, 705.
Gray, F. V. (1946). Thesis. University of Adelaide.
Gray, F. V. (1947). *J. Exp. Biol.* **24**, 1.
Gray, F. V. (1948). *J. Exp. Biol.* **25**, 135.
Gray, F. V., Pilgrim, A. F. & Weller, R. A. (1951). *J. Exp. Biol.* **28**, 74.
Gray, F. V., Rodda, H. J. & Weller, R. A. (1949). Unpublished data.
Hale, E. B., Duncan, E. W. & Huffman, C. F. (1932). *Proc. Amer. Soc. Anim. Prod.* **32**, 389.
Jarrett, I. G., Gray, F. V. & Rodda, H. J. (1949). Unpublished data.
Kick, C. H., Gerlaugh, P., Schalk, A. F. & Silver, E. A. (1938). *Bull. Ohio Agric. Exp. Sta.* no. 592.
Phillipson, A. T. (1942). *J. Exp. Biol.* **19**, 186.
Smith, V. R. (1941). *J. Dairy Sci.* **24**, 569.

THE EVAPORATION OF WATER FROM WOODLICE AND THE MILLIPEDE *GLOMERIS*

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(With Twelve Text-figures)

INTRODUCTION

The importance of adequate means of water conservation in small terrestrial animals needs no stressing and, as Wigglesworth (1946) has pointed out, the part played by the integument in this respect is of central importance since all other means of water economy are useless if the integument is freely permeable. Epicuticular waxes, investigated by Beament (1944), Lees (1947) and Lees & Beament (1948), have contributed largely to the success of insects and ticks as terrestrial animals. Little is known, however, of the water relations of other terrestrial arthropods, and the woodlice are of particular interest in this connexion since they are the only Crustaceans which have succeeded in colonizing the dry land to any extent. In so far as the water relations of this group have been investigated it is the behavioural aspect which has received most attention, e.g. Gunn (1937), and apart from the work of Lafon (1948) on *Ligia*, there has been no systematic attempt to investigate the properties of the cuticle as it affects water conservation, though the question is clearly of interest.

It appears to be the opinion of many authors, from Webb & Sillem (1906) onwards (e.g. Vandel, 1943; Cole, 1946; Hatchett, 1947—see discussion below), that woodlice can be arranged in a series from *Ligia* at the bottom, through such forms as *Trichoniscus*, *Philoscia*, *Oniscus*, and *Porcellio* to *Armadillidium* at the top, such that their power of water retention varies from least to greatest and that their distribution ranges correspondingly from wetter to drier habitats. While this may very well be so, there has been no satisfactory proof, for we have little information either about the rate of evaporation under controlled conditions, or of the microclimatic conditions in which the various species live. The work to be described was carried out in an attempt to provide information under the first head, and also in the hope that the results might add to our knowledge of the physiological problems involved when an essentially aquatic group invades the land.

MATERIAL

Seven species of woodlice and a millipede were used. The former were chosen to represent a complete range of habitat as far as humidity conditions are concerned, and their names and notes on the localities from which they were collected follow. *Ligia oceanica* Linn. is a large, littoral species of which material was obtained from Plymouth. *Philoscia muscorum* Scopoli, is a small species, confined to apparently damp surroundings such as dead leaves in woodlands and some rubbish heaps. This species was obtained from Surrey gardens and from woodlands in Devon. *Oniscus asellus* Linn. is ubiquitous and material was obtained from several sources. *Porcellio scaber* Latr. is also very common. *Cylisticus convexus* De Geer, is very patchy in occurrence, and not easy to find in large numbers. Material was obtained mainly from a limestone outcrop near Dudley in Staffordshire, known as the Wren's Nest. *Armadillidium nasatum* Budde-Lund was obtained from a greenhouse near Dorking in very large quantities, and *A. vulgare* Latr., the common pill woodlouse, was obtained from Box Hill, from rubbish heaps in Surrey gardens, and from a village near Newmarket.

The millipede, *Glomeris marginata* Koch, was included in this comparative study because it inhabits the same ecological niche as *Armadillidium vulgare*, and material of both animals was very often obtained in the same microhabitat.

In the laboratory, the animals were fed on potato and kept in 12 in. Petri dishes on damp sand together with a few large flat stones. Given clean sand, stones and food every week or so, they will live indefinitely under these conditions.

Animals for experiment were removed from the cultures and kept for 24 hr. in a small Petri dish with moist cotton-wool, but without sand or food.

METHODS

In attempting to measure the rate of evaporation from animals the clearly desirable condition that each animal shall be subjected to a constant and known humidity is by no means easy to attain. As Ramsay (1935) has shown, it is extremely difficult to determine the precise humidity conditions to which an animal is exposed when kept in a closed vessel, in 'still' air over sulphuric acid or other humidity-controlling substance. Further, a number of animals kept together in the same experimental chamber, particularly if the animals are in contact or even near to one another, will not all be under the same conditions. In order to minimize these disturbing effects an apparatus was designed in which several animals could be used at the same time but in which they could be kept separate from one another and in moving air of known temperature and humidity. The apparatus is by no means perfect, but it does reduce the two main sources of error while remaining simple in construction.

The essentials are shown in Fig. 1. The animals are kept in a long glass tube and separated from one another by a number of perforated metal disks which are all attached to a central rod. The rod and disks are made to fit snugly enough to prevent any movement of animals from one compartment to another. This 'separator tube' (*S*) is attached to a board which also carries two similar tubes each filled with calcium chloride (in the dry air experiments) through which air passes before entering the separator tube. The board with its three tubes is placed in a large tank where the temperature of the water is controlled to within 0·5° C., and air is circulated through the apparatus by a small pump (*AP*). The air is led through a copper coil in the water tank so that it takes up the temperature of the water before entering the drying tubes. Two taps in the air tube leading from the apparatus back to the air pump can be set so that the rate of output can be measured by collecting the bubbles in a graduated cylinder. (A flow-meter inserted at this point would be simpler.) A thermometer is inserted into the top compartment of the separator tube.

Animals exposed in this apparatus to moving air are probably not all subject to precisely the same speed of movement, for there are bound to be small eddies—but the air stream is slow (about 1 l./min.) and consequently the differences will not be great. It may also be objected that the animal in the top compartment is not subjected to dry air, since by the time it reaches this point the air has already passed over several evaporating animals. In fact, the amount of water vapour taken up by the air as it passes along is very small (something in the order of 0·005 mg./l.) and does not significantly affect its drying power when it reaches the later animals in the series.

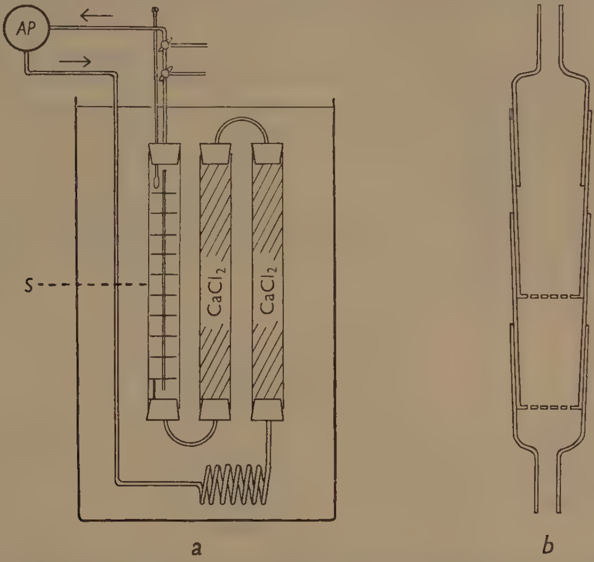

Fig. 1. *a*, diagram of the apparatus used for measuring the rate of evaporation from woodlice under controlled temperature and humidity conditions. The animals are placed one in each compartment of the separator tube (*S*) and air is circulated at 1 l./min. by the pump (*AP*). *b*, ground glass joints (B 29) with perforated glass disks, used in place of the separator tube in later experiments.

In later experiments the separator tube was replaced by a series of cones and sockets with ground glass joints (Quickfit & Quartz, B 29; see Fig. 1*b*). Each cone has a perforated glass disk sealed into the end so that when several cones are fitted together, compartments are formed in each of which one animal can be placed. This form of apparatus is clearly more versatile than the metal disk type since as many compartments as are needed can be fitted together rapidly, and furthermore there is no risk of damage to the animals as they are put in or removed.

In operation, the temperature of the water-bath and speed of air flow through the apparatus are first checked. The animals are then weighed separately, and each placed in a compartment. The apparatus is then returned to the water-bath for the prescribed period of time, after which each animal is weighed again. Any animal which shows sign of damage or which has defaecated during the experiment is discarded. For short exposures up to an hour it can safely be assumed that the difference in weight before and after exposure is almost entirely due to loss of water.

Where the rate of evaporation is being considered, the results are expressed in terms of milligrams of water per square centimetre of surface of the animal per hour. The surface area of each individual was determined by following Wigglesworth's method (1945): the cuticles of a number of weighed specimens were carefully removed and the flattened pieces drawn on graph paper by camera lucida; the surface area and weight of these individuals then enables us to find 'k' in the formula $S=kW^{\frac{2}{3}}$ where S = surface area in sq. mm. and W = weight in mg., so that the surface area of any subsequent specimen can easily be estimated from its weight. The values of 'k' found for the various species used were as follows: *Porcellio*, 12·4; *Cylisticus*, 10·8; *Oniscus*, 13·6; *Philoscia*, 10·5; *Armadillidium vulgare*, 11·5; *A. nasatum*, 11·8; *Ligia*, 12·3; *Glomeris*, 10·5.

EXPERIMENTAL RESULTS

(1) *Preliminary experiments*

Before proceeding with the main comparative work, measurements were made to find whether there was any difference in rate of evaporation between the sexes or between living and dead animals.

The estimations were carried out at 30° C. in dry air. The results showed no difference as regards sex for exposure periods of either 15 min. or an hour, and need no further comment. As regards living and dead animals, the results were rather variable, but statistical analysis showed no significant difference so far as short exposures (up to an hour) are concerned.

In view of these results, males and females were used in further work without distinction (except females carrying brood pouches which were never used), and except where the contrary is stated the animals used were living, at least at the beginning of the experiments. Animals were chosen which were of approximately the same size, and colour varieties were excluded.

(2) *The site of water loss*

It has been demonstrated by Mellanby (1934), Wigglesworth & Gillett (1936) and Koidsumi (1934) that the greater part of evaporation from insects takes place through the spiracles. Respiration in woodlice is carried out by the pleopods or 'gills' on the ventral abdominal segments. In some genera the pleopods contain bunches of tubules or 'trachea' (see Verhoeff (1920) for a detailed description), but in none is there anything so elaborate as the insect spiracular apparatus or tracheal system.

Experiments were carried out to find whether water is lost more readily in the region of these pleopods than elsewhere. Specimens of *Armadillidium*, *Porcellio* and *Ligia* were used, and they were exposed, ten at a time, in the apparatus already described, for 1 hr. at 30° C. Recently killed animals were used and they were exposed in three series: (i) uncovered, (ii) the dorsal surface covered with beeswax, and (iii) the gills covered with beeswax. The loss of weight (which can be taken as loss of water) was found in each series and from these results the loss from the dorsal surface only, the whole ventral surface, and the gills only, was calculated. Table 1 contains the results.

Table 1. *The absolute and relative amounts of water lost by woodlice from the dorsal, total ventral, and gill areas, when exposed to dry air at* 30° C.

	Dorsal surface only	Total ventral surface	Gill area
Armadillidium:			
Percentage of original weight lost	2·2	2·5	1·5
Percentage of total surface	33·6	64·6	1·8
Rate of loss/unit area	6·5	3·8	83
Porcellio:			
Percentage of original weight lost	3·2	3·7	2·9
Percentage of total surface	38·2	58·8	3·0
Rate of loss/unit area	8·4	6·3	97
Ligia:			
Percentage of original weight lost	4·2	6·4	2·5
Percentage of total surface	36·6	59·1	4·3
Rate of loss/unit area	11·5	10·9	58

If one considers the loss of water from each of the areas concerned as a percentage of the original weight of the animal, the figures show that there is no very great difference: the absolute amount of water lost from the gills, the rest of the ventral surface and the dorsal surface is of the same order. But to make a valid comparison of relative rates we need to know not only the absolute amount of water lost, but the total evaporating surface of each of the areas considered. Measurements of gill area* were made as described above, and expressed as a percentage of the total area of the animal. These figures are also shown in Table 1, and another simple calculation enables one to find the rate of loss per unit area in arbitrary units. When this is done it becomes clear that the rate of loss is very much greater from the gill area than from elsewhere, a fact which is clearly associated with the respiratory function of the gill surfaces.

The figures also appear to show that the rate of evaporation from the dorsal surface is greater than that from the ventral, a finding which if true would be rather surprising. But it is in fact almost impossible to estimate the true evaporating area of the ventral surface of a woodlouse. The figures which appear in the table show the total area, including all surfaces of the legs and so forth, whereas it is very likely that many of these surfaces are in contact with each other or so close as to inhibit evaporation from them. We can, therefore, make a generous reduction in total area of the ventral surface, and this would bring the rate per unit area up to that from the dorsal surface.

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* In this connexion 'gill area' means the area of that part of the body occupied by the gills, not the total surface of all the gill lamellae.

It is somewhat surprising to find the rate of loss from the dorsal surface as high as it is, compared with the ventral surface, for in life the ventral surface of the animal is close to the substratum which is usually moist whereas the dorsal surface is much more exposed, and one might have expected to find water-retaining properties present in the dorsal surface to a much greater extent than in the ventral.

If a comparison is made between the three species considered, it is clear that the rate of loss is greatest in the littoral-living *Ligia* and decreases through *Porcellio* to the drier-living *Armadillidium*. The figures for *Ligia* gill area are an exception to this, and they may well be due to the much greater proportion of the total area occupied by gills in that animal.

(3) *The relation between rate of evaporation, temperature and humidity*

The rate of evaporation in dry air was determined at 10° intervals from 10° to 60° C., for all the species mentioned above. There were two series of experiments, in the first the animals were exposed for 15 min., and in the second, for an hour. At least ten animals and frequently many more were exposed at each temperature. The rate of evaporation per unit area was calculated for each animal and the mean calculated for the group.

The results for exposures of 1 hr. are shown in Table 2, and a selection of them has been graphed in Fig. 2. They show that there is an increase in the rate of evaporation as temperature rises, and that this increase is greater at higher than at lower temperatures. It will also be noticed that the rate of evaporation from *Philoscia* is higher at all temperatures than is that from *Porcellio*, while the rate for *Armadillidium* is lower still. *Glomeris*, it will be seen, gives a curve similar in general shape to those given by woodlice. Fig. 2 also shows the curve for the cockroach *Blatella*, derived from Wigglesworth's data (1945), and the shape of this curve can be seen to differ significantly from the woodlouse curves. It shows no rise in the rate as temperature increases up to about 35° C. after which the rate rises very steeply. It is now well

Table 2. *Rate of evaporation of water in mg./cm.²/hr. when exposed to dry air for an hour at various temperatures*

	Temperature in ° C.					
	10	20	30	40	50	60
Armadillidium	1·5	1·5	2·7	4·5	6·5	9·5
Porcellio	1·0	1·2	3·0	6·0	10·0	17·0
Cylisticus	1·0	3·5	7·0	9·0	13·0	18·5
Oniscus	2·0	2·5	4·2	7·0	11·5	18·5
Philoscia	3·0	4·5	5·7	9·0	14·0	19·5
Ligia	2·0	4·2	7·0	12·5	15·0	19·7
Glomeris	1·2	2·7	5·5	9·0	14·5	21·0

known that the shape of the curve for insects is attributable to a change in phase of a very thin waxy layer in the epicuticle at the 'critical' temperature, above which the wax does not prevent the passage of water to anything like the extent which it does below this temperature.

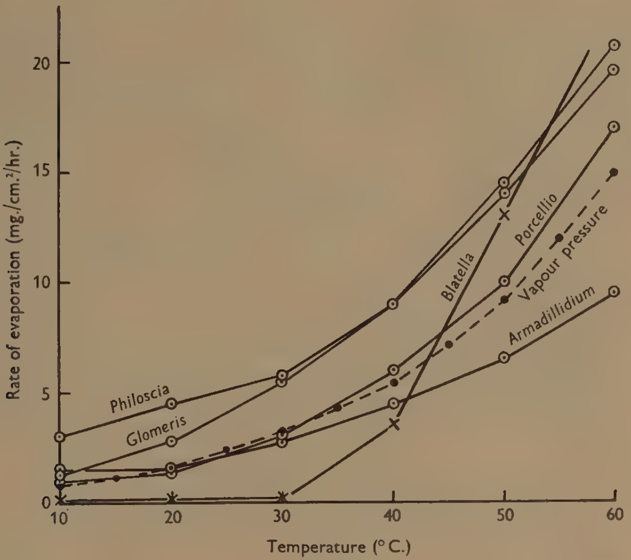

Fig. 2. The rate of evaporation of water from various woodlice, the millipede *Glomeris* and a cockroach (*Blatella*) at temperatures from 10° to 60° C. in dry air; the animals exposed for 1 hr. periods.

Fig. 2 also shows a curve labelled 'vapour pressure'. This represents, on an arbitrarily chosen scale along the ordinate, the water-vapour pressures of saturated air at the temperatures along the abscissa. (This is, of course, the same as a saturation deficit/temperature curve for dry air.) The striking similarity between the shape of this curve and the shape of the evaporation curves for woodlice suggests a close relationship. If the relation is a linear one, as seems likely, then rate of evaporation graphed against saturation deficit instead of temperature, should give a straight line. In Fig. 3 this has been done, and it is clear that an approximately linear relationship does exist for both woodlice and *Glomeris* (but not, it will be noticed, for the insect *Blatella*). The significance of the position of the various curves on this graph will be further discussed below.

We may now consider the corresponding results for exposures of 15 min. These are shown in Table 3 and a selection is graphed in Fig. 4. Here again the same general picture appears, the most significant difference being the general increase in rate of evaporation shown over those for an hour's exposure. The curve for *Porcellio* for 1 hr. exposure has been included in Fig. 4 for comparison, and the figures for other species show the same effect. The reason lies, of course, in the fact that water is evaporated at a greater rate during the first quarter than during the remaining quarters of a complete hour's exposure. This effect may itself be caused by a drying of the outer layers of the integument which is thus made less permeable, but whatever its cause it is clearly of advantage to the animals since it acts as a regulatory mechanism, reducing the rate of loss of water at a time when conservation is most needed.

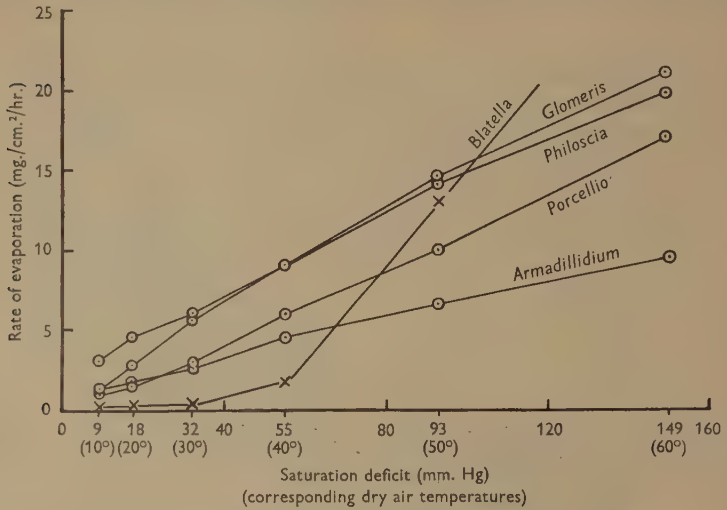

Fig. 3. The same data as in Fig. 2, but with saturation deficit expressed along the abscissa on a linear scale.

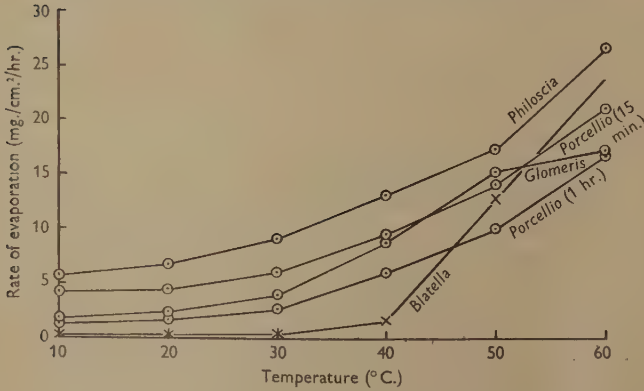

Fig. 4. Rate of evaporation from woodlice and from *Glomeris* and *Blatella* at various temperatures, the animals exposed for 15 min. periods.

Table 3. *Rate of evaporation of water in mg./cm.^{2}/hr. when exposed to dry air for 15 min. at various temperatures*

	Temperature in ° C.					
	10	20	30	40	50	60
Armadillidium vulgare (i)*	6·0	7·0	8·7	12·0	13·0	13·5
A. vulgare (ii)*	4·5	5·2	6·2	13·5	13·7	14·0
A. nasatum	3·0	4·5	5·7	11·5	15·5	9·0
Porcellio	4·0	4·5	6·0	9·5	14·0	21·0
Cylisticus	4·5	7·5	12·0	15·0	17·0	23·0
Oniscus	4·5	4·5	6·5	8·5	13·2	22·5
Philoscia	5·6	6·7	9·0	13·0	17·0	26·5
Ligia	5·5	8·5	11·5	17·0	19·5	22·0
Glomeris	1·7	2·5	4·0	8·7	15·2	17·0
Blatella†	0·1	0·1	0·2	3·5	13·0	—

* Group (i) consisted of specimens from the Hogs Back, Surrey; group (ii) from near Newmarket, Suffolk.

† Data from Wigglesworth (1945).

There is one interesting exception to this general effect however, and that is shown by *Glomeris*, whose rate of evaporation during the first quarter of an hour is maintained (except at 60°) during the rest of a complete hour's exposure. The explanation of this is by no means clear, though its effect is to place *Glomeris* at or near the top of the scale for 1 hr. exposures, though it is one of the lowest in the scale (particularly at low temperatures) for 15 min. exposures.

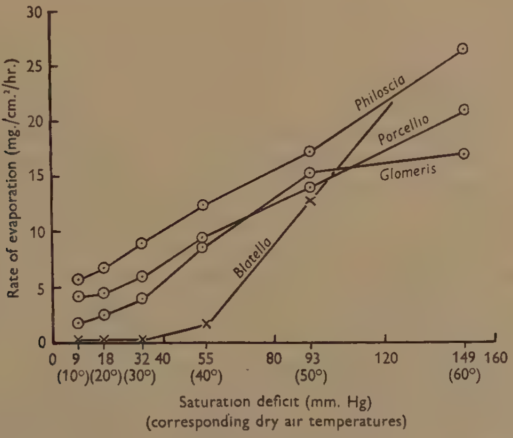

Fig. 5. The same data as in Fig. 4, but saturation deficit expressed along abscissa on a linear scale.

In Fig. 5 the results for 15 min. exposures are graphed against saturation deficit, and again an approximately straight line relationship appears (cf. *Blatella*) except for *Glomeris*, where the rate falls off significantly above 50° C.

It will have been noticed that *Armadillidium* appears in neither of the last two graphs. This is because its behaviour, like that of *Glomeris*, is anomalous at higher temperatures. Fig. 6 shows this anomalous behaviour for *A. vulgare* (from the Surrey chalk in one series and from Suffolk in another) and for *A. nasatum*. It is clear that in all three species the usual saturation deficit curve is followed reasonably well up to 40° C., above which there is a strong falling off. This takes the form of an absolute drop in rate in *A. nasatum* between 50° and 60° C., and a more or less constant rate in *A. vulgare* from 40° to 60° C. (a relative drop compared with the rate if it followed the S.D. curve). The significant feature is certainly the fact that there is a strong falling off, the extent to which this takes place is of lesser importance.

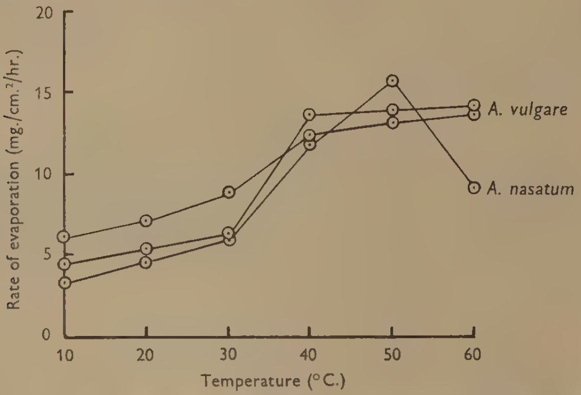

Fig. 6. The rate of evaporation from two species of *Armadillidium* at various temperatures.

Now since the effect is observed in both species of *Armadillidium* and in *Glomeris*, all of which are able to curl into a ball, the power to do so seems to offer a likely source of explanation of the effect. Attempts were therefore made to discover whether these species do curl up at high temperatures and to measure the rate of loss when they are kept open. Observation showed that they do indeed curl up above 40° C., but to a variable extent and for variable periods, for they were killed by temperatures just above 40° and after death they always relaxed slightly; and experiment seemed to show that even when kept flat by being encased in gauze envelopes, the animals lost water less rapidly at 50° and 60° C. than would be expected. The technique used for keeping them flat, by enclosing them in wire-gauze envelopes, was by no means satisfactory however, for it was never possible to be quite sure that scratching of the surface or other damage had not occurred; so that the theory of restriction of loss by curling, though apparently quite likely, cannot be said to have been proved, and some other method of keeping the animals open must be found.

It has been shown by the experiments described above that, with certain reservations, the rate of evaporation of water bears a linear relation to saturation deficit when the air is dry. It follows from this that temperature *by itself* has no effect upon rate of evaporation, and it should be possible to demonstrate this experimentally by keeping the saturation deficit constant while varying the temperature. The results of such an experiment would also tell us whether the relation between saturation deficit and rate of evaporation holds not only when the air is quite dry, but at other relative humidities as well.

Solutions of sulphuric acid and water were made up for each 5° C. interval in temperature from 20° to 45° C., such that each would give a saturation deficit of 17·5 mm. Hg. This meant having a relative humidity of 0% at 20° C. and one of 76% at 45° C. Wash bottles containing pummice and the necessary solution then took the place of the calcium chloride tubes in the apparatus, and measurements of loss of weight were made as usual. *Porcellio scaber* was the animal used, and the results are shown graphically in Fig. 7. Exposures were for both 1 and 2 hr. periods, and the results showed a constant rate of loss for all temperatures up to 35° C. For temperatures higher than this the rate of loss increases slightly for 1 hr. exposures, and to an even slighter extent for 2 hr. exposures. There are two contributory causes for this rise, one theoretically necessary, the other of experimental origin. The latter is probably of greater effect and it arises from the fact that at high temperatures the R.H. has to be rather high, so that after the weighed animals have been put into the apparatus, the humidity takes a little time to reach the desired level. The animals therefore respond to too dry conditions and the initial rate of loss is high. The second and theoretically necessary cause lies in the fact that even ideally the rate of evaporation is not precisely proportional to S.D., for there is an increase approximately in proportion to the square of the absolute temperature, due to the increased rate of diffusion of gases. Making allowances then for these two factors it can safely be said that the effects of temperature upon the evaporation of water from woodlice are such as can be deduced from the laws of gaseous diffusion, and that there is no evidence of an effect of temperature upon the permeability of the integument.

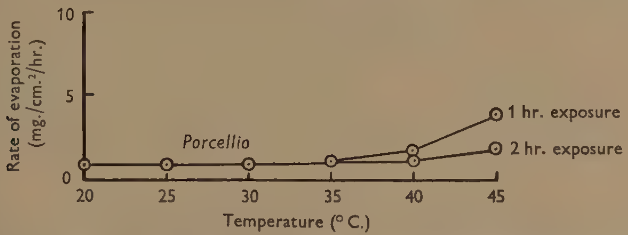

Fig. 7. The rate of evaporation from *Porcellio* at various temperatures, the saturation deficit being kept constant at 17·5 mm. Hg, by varying the relative humidity.

We must now examine the curves for *Ligia oceanica* which were not included in the graphs discussed above. They are shown in Fig. 8, and it is clear that for both 15 min. and 1 hr. exposures the rate of increase in evaporation falls off significantly above 40° C. This is shown clearly when saturation deficit is used as the abscissa (broken lines). Now *Ligia* is considerably bigger than any of the other woodlice used, and weights of 0·9 g. are by no means uncommon, whereas a fairly large *Armadillidium* weighs only 0·2 g., and a *Philoscia* about a fifth of this. Furthermore, the surface of *Ligia* always appears to be slightly moist, and it seems not unlikely that *Ligia* is sufficiently large and has a sufficiently permeable cuticle, to be able to cool itself to such an extent as significantly to reduce its rate of evaporation at high temperatures. The absolute rate of evaporation at all but the highest temperatures is a good deal higher in *Ligia* than in any other species studied, a fact which lends support to the cooling theory, and experiments at present in progress have already shown that the internal temperature of *Ligia* in dry air can be held at several degrees lower than the external temperature.

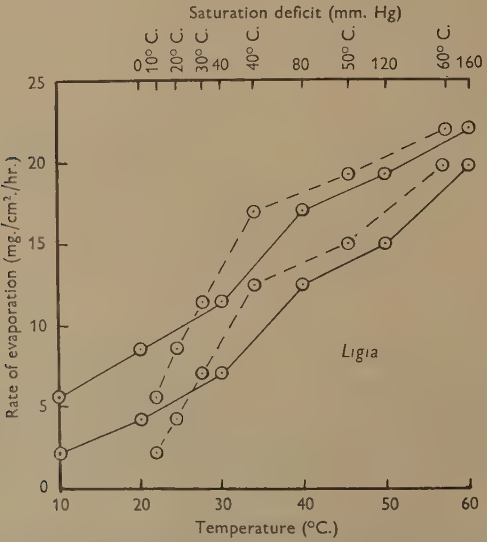

Fig. 8. The rate of evaporation of water from *Ligia* in dry air in relation to temperature on a linear scale (continuous line) or to saturation deficit on a linear scale (broken line); for exposures of both 15 min. and 1 hr.

(4) *The effect of humidity upon upper lethal temperatures*

Besides its indirect effect through saturation deficit and rate of evaporation, temperature has of course a direct effect both on general metabolic processes and, presumably, on distribution. Little is known about the range of temperature tolerance in woodlice, and nothing, so far as the writer is aware, of the relation of humidity and temperature tolerance. Here again our knowledge of woodlouse physiology lags behind that of insects, for the effect of humidity on the thermal death points of insects has been known for some time from the work of Mellanby (1932) and others.

The present series of experiments was designed to find the highest temperature which woodlice can tolerate for periods of either 15 min., 1 or 24 hr., in 0, 50 and 100% R.H. The animals were exposed, ten at each set of conditions, in the apparatus already described, and air of the required humidity was bubbled through at the rate of 1 l./min. The highest tolerable temperature was determined to the nearest degree, very little variation amongst individuals being found in this respect. Almost invariably if half the animals were alive after exposure to x° C., then all the animals in another batch would be dead at $x+1$° C., and alive at $x-1$° C.

The results of these experiments are shown in Table 4, and a selection is represented graphically in Fig. 9. It is clear from these results that for any one species (with the exception of *Ligia*), the lethal temperatures for a 15 min. exposure are substantially the same at all three humidities used (though slightly lower at 100% R.H. than in drier air, and slightly higher at 50% R.H. than at other humidities). For exposures of 1 hr., the findings are parallel, but all the temperatures are slightly lower (from 1° to 5° C.). For exposures of 24 hr., however, the picture is very different. Here the humidity has a profound effect, for in dry air the lethal temperature is about 20° lower than it is in saturated air, and even at 50% R.H. the lethal temperature is very greatly depressed.

Table 4. *The highest temperature which various species of woodlice and other arthropods can tolerate at different humidities for various periods of time*

Period of exposure ...	15 min.			1 hr.			24 hr.		
Relative humidity (%)...	0	50	100	0	50	100	0	50	100
Armadillidium vulgare	43	46·5	42	41	42	40	18	22	37·5
A. nasatum	42·5	45·5	41	41	41·5	40	11	17·5	37·5
Porcellio	42·5	43	38	39	39·5	37·5	13	17·5	36
Oniscus	40	41·5	37	34	36·5	33·5	9	15	31·5
Cylisticus	—	—	—	35·5	36	37·5	12·5	13·5	35
Philoscia	—	—	—	32·5	36·5	34	9	13	30·5
Ligia	41·5	40	34·5	39	35	32·5	9	—	29
Glomeris	—	—	—	42	42·5	42	27	33·5	38

Period of exposure ...	1 hr.				24 hr.			
Relative humidity (%) ...	0	30	60	90	0	30	60	90
*Pediculus**	46	46	46	46	33	35	37	38
Xenopsylla larvae*	39·5	39·5	39·5	39·5	22	27	32	37

* Data derived from Mellanby (1932).

In the main then it can be said that the animals are dying from high temperature at the short exposures (of 15 min. and an hour) but that they are drying out and dying from desiccation during the long exposure (except at 100% R.H. of course). The lethal temperatures found at 100% R.H. probably represent the true tissue tolerance, and it is significant that these do not differ widely with different periods of exposure. During brief exposure periods it appears that there is some benefit to be derived from evaporation (and hence cooling) in non-saturated atmospheres; the effect is rather greater at 50% R.H. than in completely dry air where the rate of evaporation is probably so great that the cooling effect cannot be maintained over the whole period.

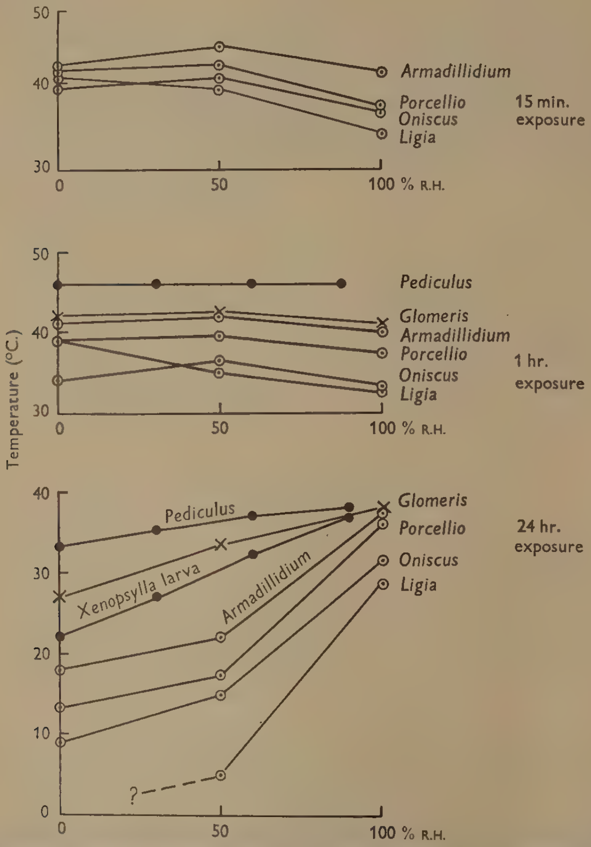

Fig. 9. The highest temperature which various woodlice, *Glomeris* and the insects *Pediculus* and *Xenopsylla* (larvae) can tolerate, at different humidities and for various periods of time.

Ligia behaves somewhat differently from the rest, and the differences are again very readily explained by its greater size and therefore greater water evaporating capacity. At 15 min. and 1 hr., there is in all but saturated atmospheres a cooling effect which raises the apparent temperature tolerance above the true tolerance of the tissues. In 24 hr. exposures, the temperature tolerated is much lower at 50% R.H. than it is when the air is saturated, and this is to be expected in an animal whose water-conserving powers are so feeble.

In Fig. 9 the woodlice are also compared with *Glomeris* and with the insects *Pediculus* and *Xenopsylla*, the data for the insects being taken from Mellanby (1932). The various species of woodlice stand in approximately the same order as they do in respect of rate of evaporation; that is *Armadillidium* has the highest temperature tolerance and the lowest rate of evaporation, *Ligia* has the lowest temperature tolerance and the highest rate of evaporation, the remainder being intermediate. *Glomeris*, it will be noticed, has a 'true' temperature tolerance just above the highest woodlouse, and in 24 hr. exposures its capacity to resist high temperatures does not fall to nearly the same extent as that of the woodlice, and in this respect it resembles the insects. Both of the insects to which Fig. 9 refers have an upper temperature limit which is independent of humidity for short exposures but, like woodlice, they show a drop in temperature tolerance in dry air for long exposures, though the drop is not so great as it is in woodlice.

(5) *Recovery after desiccation*

We have seen that water is easily lost from woodlice, and it remains to find out whether they can re-absorb it if they are returned to suitable conditions after desiccation. It was shown by Verhoeff (1920) that land isopods can absorb liquid water by means of the uropods, which convey it to the pleopods or the rectum. Presumably such liquid water can also be imbibed through the mouth. What is uncertain is whether the animals can absorb water vapour from damp air, as a number of insects and ticks can.

This question was investigated by subjecting individuals of various species to desiccation either for 45 min. at 0% R.H. and room temperature (15° ± 2° C.) or to 50% R.H. for 23 hr. at the same temperature, after which they were kept without food and again at the same temperature in small Petri dishes with moist cotton-wool where the air was presumably saturated with water vapour, or in desiccators at 98% or 95% R.H. They were weighed before desiccation, immediately after, and then at approximately 2 hr. intervals for the first 6 hr. and daily thereafter for 8 or 10 days. All of those which were kept at 95% R.H. after desiccation lost weight and ultimately died. Those which were kept at 98% showed somewhat erratic behaviour, and of those kept in saturated air, all survived and the great majority gained weight.

In Fig. 10 an attempt is made to show the changes in weight of these groups. In each graph the thin horizontal line represents the weight immediately after desiccation, and the thick line represents the changes in weight shown by the animals during the experimental period expressed as a percentage of the post-desiccation weight. The thick vertical line at the commencement of each graph represents the weight lost during desiccation, but this does not apply to column *C* where the animals were desiccated for 23 hr. and the amount of weight lost was too great to be included conveniently in the graphs as set out: losses of 50% of the post-desiccation weight were common under these conditions. Column *A* refers to animals which were desiccated in dry air for 45 min. and subsequently allowed to recover in 98% R.H. Those represented in column *B* suffered the same desiccation, but recovered in 100% R.H., while those in column *C* were desiccated for 23 hr. at 50% R.H. and allowed to recover in 98% R.H. Six animals of each species were treated in each of the conditions represented, and the relevant curve shows the mean behaviour of the six animals. Where the behaviour was too variable for this treatment three curves are shown on the one graph representing the two extremes and a central example of the behaviour. It should be emphasized that all these animals (except *Philoscia* in column *A* and *Glomeris* in *A* and *B*) were still alive at the end of the experimental period, even though many of them lost weight.

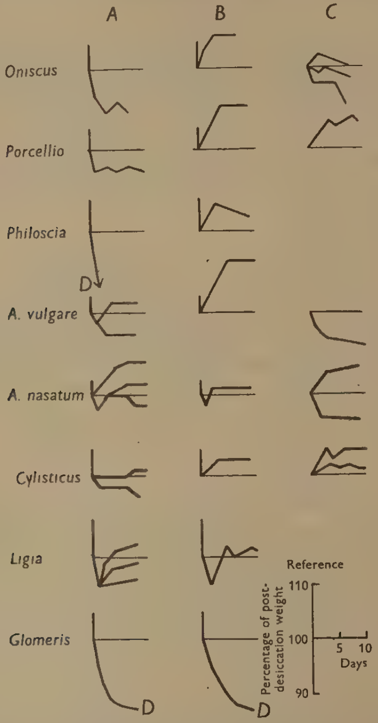

Fig. 10. Change in weight after desiccation in woodlice and *Glomeris*. Thick vertical line: the extent to which weight was lost during desiccation; thin horizontal line: immediate post-desiccation weight. *A*, animals desiccated for 45 min., recovery in 98% R.H.; *B*, similar desiccation, recovery in 100% R.H.; *C*, animals desiccated for 23 hr. at 50% R.H., recovery in 98% R.H. All at room temperature (15° ± 2° C.).

No species was capable of recovery in 95% R.H. and no graphs referring to this condition are shown. The graphs in column *A* show that most species lose weight in 98% R.H. after desiccation. *Armadillidium*, however, appears to be just about able to hold its own in this humidity, and in many instances the weight gained is actually more than the original weight lost during desiccation. *Ligia*, surprisingly, manages to survive and increase in weight after an initial loss, though the degree to which weight is recovered is very variable. *Glomeris* is quite incapable of regaining weight and loses rapidly even in 100% R.H.

Column *B* shows that all species are capable of surviving and gaining weight in 100% R.H. after desiccation, though it is a doubtful point as to whether this is by the absorption of water vapour or of liquid water, for it was found to be impossible to keep saturated air without small droplets condensing on surfaces. Even if kept at a constant temperature small energy systems set up inside the vessel lead to slightly different conditions in one part of the vessel from others and so to condensation.

Column *C* shows that after desiccation for 23 hr. at 50% R.H. rather more animals are capable of recovering weight in 98% R.H. than in the corresponding series in column *A*, but the results are irregular, and sufficient material was not available to experiment with all the species. The parallel series of experiments showing recovery in 100% R.H. after desiccation for 23 hr. at 50% was not carried out on all species. The two species which were used, *Oniscus* and *Porcellio*, both showed good recovery of weight in 100% R.H., and it can safely be assumed that the rest would have done, for as mentioned above there is plenty of liquid water about.

In general then, it can be said that after suffering water loss by desiccation, all the species of woodlice studied are capable of recovering at least some of the water lost if they can reach saturated air, and that *Armadillidium* and *Ligia* but not other species can do so from a relative humidity of 98%. On the other hand, no recovery of water takes place from 95% R.H. or below. The behaviour is very variable, but perhaps this is only to be expected since the animals were subjected to treatment very near the limit of their tolerance.

Having shown that at least some animals are capable of absorbing water vapour from unsaturated air, the question remains as to whether this is a property of living animals only, or whether the same phenomenon can be observed in dead ones. Specimens of *Oniscus* and *Armadillidium vulgare* were killed with ammonia vapour and subjected to the same desiccation as living ones, after which they were allowed to recover at 100 and 98% R.H. In saturated air the behaviour of the dead animals was variable, they frequently showed a gain in weight, but they never gained as much as the most successful living ones, and occasionally they remained steadily at the same weight (until putrefaction began to have its effect). This variable behaviour is probably nothing more than a result of the fact that in a closed vessel where there is a free water surface, the condensation of water droplets takes place in an unforeseeable way and leads to extremely doubtful results. In 98%, no dead animal recovered weight at all, though in *Oniscus* the amount lost was smaller than that lost by living animals. The results are shown in Fig. 11.

A further point of interest in this connexion is that an animal which died during the recovery period invariably lost water very rapidly before doing so. It is as though 98% R.H. is very near the lower limit of humidity which *Oniscus* can withstand: in some individuals the metabolic machinery concerned is tipped slightly over in the wrong direction, all control is lost and the animals lose water rapidly until exhausted, whereas in others, a precarious equilibrium is just maintained and water gained just balances water lost.

It has so far been assumed that relative humidity is the factor involved in determining whether a woodlouse shall or shall not be able to regain lost water from the atmosphere. This is not necessarily true, for the limiting factor might be saturation deficit, and the following experiment was designed to discover which of the two measures is involved.

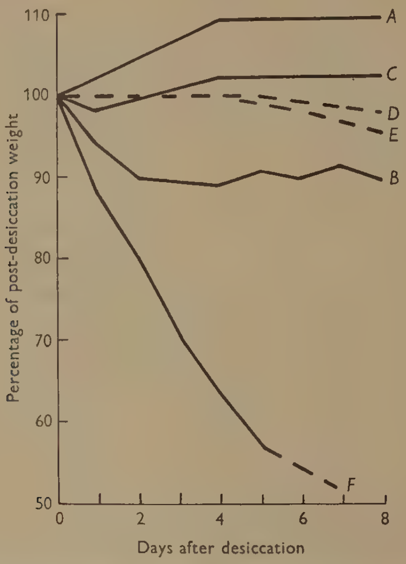

Fig. 11. A comparison of post-desiccation weight changes in living and dead woodlice. Solid lines: live animals; broken lines: dead animals. *A*, live *Oniscus* in saturated air; *B*, the same in 98 % R.H.; *C*, live *Armadillidium* in 98 % R.H.; *D*, dead *Oniscus* and *Armadillidium* in saturated air: *E*, the same in 98 % R.H.; *F*, *Oniscus* in 98 % R.H. which died after 5 days.

Specimens of *Armadillidium vulgare*, a species able to hold its own when allowed to recover from desiccation at 17° C. and 98 % R.H., were allowed to recover in the same relative humidity, but at either 4° or 30° C., and their weights plotted. Now if saturation deficit is involved, an animal which holds its own at 17° C. should be able to recover very well at 4° C., since the saturation deficit is only 0·12 mm. Hg at 4° C. whereas it is 0·29 mm. at 17° C. At 30° C. and the same R.H. the saturation deficit is 0·64 mm., more than five times the value at 4° C., and one would expect the animal to lose water rapidly. However, as the graphs in Fig. 12 show, there is little significant difference between the behaviour of the animals at all three temperatures, and we are safe in inferring from this that relative humidity is the limiting factor.

DISCUSSION

It will be convenient to discuss: first, the extent to which the present results agree with what is already known of the water relations of woodlice; secondly, their significance when compared with parallel results for insects; and thirdly, any new light which they throw upon the subject of woodlouse ecology.

As stated above, very few measurements have been made of the rate of evaporation from woodlice. Allee (1926) showed that *Oniscus* and *Cylisticus* if kept singly lose water more rapidly than if they are allowed to bunch, but his findings are qualitative only. Numanoi (1934), working with *Ligia exotica* only, kept them suspended in air over sulphuric acid at 25° C. and found the time taken to die at different relative humidities varied from 5 hr. at 2% R.H. to 16–20 hr. at 76% R.H., and that the amount of water lost before death occurred was about 13% at all humidities save 100%. These figures do not conflict with those found for *L. oceanica* in the present work, but they do not tell us much about the nature of the relation between humidity and evaporation beyond the qualitative fact that in drier air evaporation is faster.

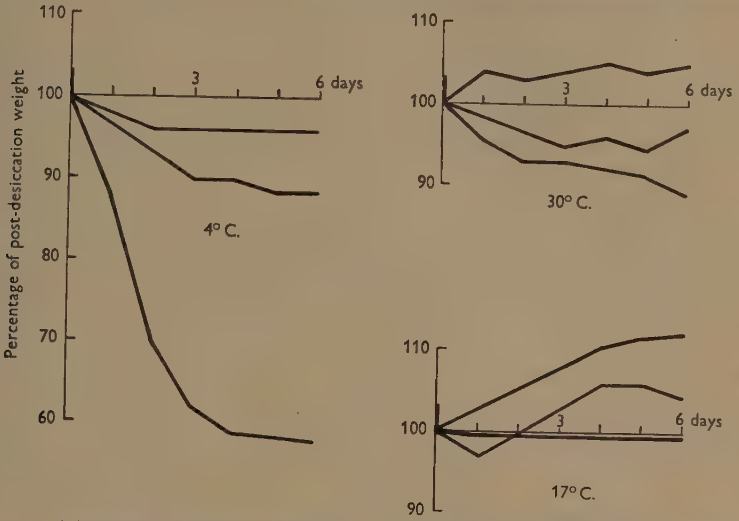

Fig. 12. The effect of varying the temperature at which *Armadillidium* is allowed to recover after desiccation for 45 min. in dry air. For explanation see text.

Miller (1938), working on the isopods of the San Francisco Bay region, exposed a number of species to different humidities at different temperatures (four temperatures and eighteen combinations of temperature and humidity in all) in desiccators, and noted the times taken to die. Expressing the results in terms of saturation deficit he obtained the best linear relationship by using an exponential equation of the form $T = ae^{-mS}$, where T = survival time, a and m are constants, and S = saturation deficit. For *Ligia* however, he found that a good straight line fit was obtained if the reciprocal of T were plotted against the saturation deficit. It should be noted, however, that his animals bunched in the desiccators and were therefore not exposed to the humidities arranged. Rate of evaporation is, of course, not being measured in an experiment of this sort (even under ideal conditions), for we do not know how survival time is related to rate of evaporation, so that a simple relation between survival time and saturation deficit is not necessarily to be expected. Miller's results therefore, do not really conflict with the present set.

Waloff (1941) found that no woodlouse could live indefinitely (over a month) without food in any humidity below saturation, although *Armadillidium* lived from 2 to 10 days in a relative humidity of 93%. *Porcellio* did rather worse than *Armadillidium* at each humidity, and *Oniscus* did worse still; results which are supported by the present experiments. She also measured the percentage weight lost by evaporation from *Oniscus* at 0, 50 and 75% R.H. and at room temperature, and found, as Numanoi did for *Ligia*, that evaporation is greater in drier air. (Her conclusion that the 'loss of water by evaporation is proportional to time and to the percentage of relative humidity' is, however, unjustified, for her figures do not even show a linear relationship between rate of evaporation and R.H. let alone a proportionality.) She also kept desiccated *Oniscus* in saturated air, and found that the animals usually gained in weight. The same results were found for all the species used in the present work, which is not surprising since there are bound to be droplets of water available.

Heeley (1941) found difficulty in maintaining cultures of various isopods in the laboratory until he found the right humidity for each species. These humidities varied from 84% R.H. for *Trichoniscus pusillus* to 65% for *Armadillidium vulgare*. It is difficult to say just what these results mean, for the humidity in the Petri dishes was controlled by dropping water on to filter-paper from time to time and measured by small paper hygrometers. The animals were also provided with fresh moist carrot, so that the actual humidity conditions to which they were subjected may have been very different from those measured by the paper hygrometers. Nevertheless, the fact that the air in these Petri dishes was by no means saturated does suggest that in nature the animals can survive in less than saturated air provided moist food is available. A study of the amount of water obtained by land isopods from their food might show interesting results.

In the present work no difficulty at all was experienced in obtaining good humidity conditions for keeping and rearing isopods of various species—in fact they all seemed to thrive in saturated air, or at least in air exposed to free water surfaces, and Miller (1938) had a similar experience.

We may now compare the present results with the situation as it is known in insects. It has been shown that, with a few exceptions in certain conditions, the rate of evaporation from isopods bears a straight-line relationship to the humidity expressed as a saturation deficit. In insects as Mellanby (1935) has shown, the same principle is at work, but, as we now know, the effect of factors other than saturation deficit upon the rate of evaporation is often so great as to reduce the original relation to insignificance. The two main reasons why saturation deficit and evaporation are not simply related in insects are (i) that the greater part of the loss takes place through the spiracles, which can be closed, and (ii) that there are relatively impermeable waxes in the epicuticle which depress the evaporation very greatly unless their critical temperature is passed. Now the fact that in woodlice the saturation deficit-evaporation relation is linear over a wide range of temperature suggests strongly that there is neither control of water loss from respiratory surfaces by a mechanism such as the spiracles of insects, nor a layer in the integument offering serious resistance to the evaporation of water. On the other hand, water does evaporate more rapidly from some species of woodlouse than from others—and there must be differences in their cuticles to account for this. Why is the cuticle of *Armadillidium* less permeable than that of *Philoscia* for instance? This aspect of the problem deserves further study—it has not been considered in the present work.

Lafon (1948) has come to a similar conclusion from work on *Ligia*. He showed that at 25° C. and in dry, still air, specimens abraded with alumina dust did not lose water more rapidly than the unabraded control specimens, and further that the incidence of death did not affect the course of water loss during the exposure period.

In woodlice the area from which the greatest evaporation takes place (if the latter is measured per unit area) has been shown to be the pleopods, but the absolute amount of water evaporated is no greater from there than from the rest of the ventral surface, or from the dorsal surface. In insects, something like 60 or 70% of the water evaporated escapes through the spiracles—and if this were expressed as a rate per unit area (of spiracular opening) it would obviously be very high indeed. The difference then lies in the fact that in woodlice the respiratory area is not protected against evaporation by spiracular mechanisms, neither is the rest of the surface protected by impermeable waxes.

It might be considered surprising to find no difference in the rate of evaporation from living and dead woodlice. But it must not be forgotten that this finding refers to relatively short exposures, of 15 min. or an hour; for such short periods (recalling that evaporation takes place from the whole surface as well as from the pleopods, and that there is no control of evaporation even from the latter) there is no reason why evaporation should be cut down in dead specimens. The finding is confirmed by the general smoothness of the curves relating rate of loss to saturation deficit, for at some temperatures the animals were alive all the time, at others only part of the time, and distinct breaks in the curves could be expected if death had altered the rate to an appreciable extent. Similar results were also obtained by Lafon (1948) for *Ligia*. For long periods such as 24 hr. no comparisons have been made.

In spite of these differences between insects and woodlice there are certain similarities in water relations. One is the depression of the upper temperature limit by low humidities in long exposures, and it indicates that in some insects, as in woodlice, there is a significant loss of water despite the wax layer. There are, however, insects (e.g. *Tenebrio* larvae, *Xenopsylla* adults (Mellanby, 1932)) where there is no such depression even at 0% R.H. for 24 hr. and this is testimony to the very effective powers of water conservation which they possess, powers which cannot be matched by any woodlice.

Some species of insects, notably *Chortophaga* (Ludwig, 1937), *Tenebrio* (Mellanby, 1932), *Xenopsylla* pre-pupae (Edney, 1947), can absorb water vapour from unsaturated atmospheres. This property is also possessed by ticks (Lees, 1946). So far as is known the faculty is confined to insects which may be exposed to very dry habitats, and it has clearly helped to open such habitats to their advance. The present work has shown that woodlice are for the most part incapable of coming into equilibrium even with such high relative humidities as 98%, and this must be a powerful limiting factor in their distribution.

On the other hand, we have evidence from the present work that some woodlice are capable of suffering very considerable water loss without dying, in some cases as much as 50% of the weight can be lost in this way without killing the animals. In normal conditions woodlice subjected to such treatment would, one supposes, subsequently regain their weight either by absorbing liquid water or by feeding or both, so that the capacity for withstanding great losses may be regarded as being of advantage to them in terrestrial existence.

A corollary to this is suggested by the temperature-tolerance curves which show that a higher temperature than the true tissue tolerance may be withstood for short periods, an advantage which is likely to be due to the cooling effect of a rather rapid loss of water.

Taken as a whole the present results show, in the species studied, a considerable range both as regards temperature tolerance and rate of evaporation. The species can be arranged in a series as regards rate of evaporation per unit area, with *Armadillidium vulgare* at the top showing the lowest rate and *Ligia* at the bottom showing the greatest. They can be arranged in the same series in respect of temperature tolerance, *Armadillidium vulgare* showing the highest tolerance. This strongly suggests that the animals are adapted for terrestrial existence differentially and according to the series mentioned above. We now know that this is certainly true so far as resistance to evaporation goes, but the other side of the picture, the measurement of conditions in which the various species do actually occur, has still to be made.

Miller's (1938) work on the terrestrial isopods of San Francisco Bay, in so far as he used the same genera as those in the present work, suggests a similar order in resistance to drought and as regards actual habitat. But it is little more than a suggestion as regards habitat, for no microclimatic measurements were made, and moistness was estimated by general impression and by height above the sea. Cole (1946), in the course of a useful study of the Cryptozoa of an Illinois woodland, did measure temperature and soil moisture under experimental boards where woodlice occurred and correlated their aggregation and vertical migration with these factors. But as he remarks, 'It will be necessary to measure microclimatic factors actually within the cryptozoic niche' in future work.

Hatchett (1947), after studying the biology of the isopods of Michigan, also correlates distribution with moistness of the environment, but supports the statement with no microclimatic data.

It may also be worth pointing out that the distribution of the common woodlice in this country is by no means certainly related to moisture: for instance *Armadillidium*, *Glomeris*, *Porcellio*, *Oniscus* and *Philoscia* have all been found abundant in a single rubbish heap in Surrey.

Glomeris, which was treated together with woodlice in the present work, is interesting in that it shows some similarities to and some differences from them as regards its water relations. Thus its evaporation-saturation deficit curve shows the same general linear relation as do those of woodlice, and the absolute rate like that of the woodlice is a good deal higher than most insects. On the other hand, its temperature-tolerance curves show on the whole a higher tolerance than woodlice, which suggests that it may be nearer the insects in this respect. Its powers of recovery after desiccation, however, are worse even than those of woodlice.

The general similarity between the water relations of woodlice and *Glomeris* throws additional light on the findings of Lafon (1943) that millipede cuticle resembles isopod cuticle in having a high calcium and low chitin content. He also showed that the other large myriapod group, the centipedes, have a cuticle which is more insect-like in gross chemical content, being much lower in calcium and higher in chitin content than the millipedes. It will be interesting to see whether the centipedes are more insect-like in their water relations than the millipedes.

In conclusion, we now know that woodlice can be considered as fairly simple physical bodies in so far as evaporation from them is concerned, and although no direct evidence is offered in the present work on the composition of the integument, there is a very strong suggestion that it lacks the waxes characteristic of insect cuticles. (It is hoped to present direct evidence on this point in a further publication.) The relatively permeable nature of their integuments limits their distribution on dry land, but some genera such as *Armadillidium* would appear to be better adapted in this respect for terrestrial existence than others such as *Philoscia*.

What we still need to know as regards the effect of water relations on woodlouse distribution is the precise conditions, of temperature and humidity in particular, to which the various species are subjected in their natural habitats.

SUMMARY

1. Comparative studies have been made on seven woodlice and the millipede *Glomeris* to determine: (i) the site of water loss, (ii) the effect of temperature and humidity on the rate of evaporation of water, (iii) the effect of humidity on their upper temperature tolerance, and (iv) their capacity to regain water after desiccation.

2. An apparatus was constructed in which several animals can be exposed simultaneously but separately to a slowly moving stream of air of any required humidity and temperature.

3. There is no difference between the sexes as regards rate of water loss, and no difference was found in this respect between living and dead animals for exposures up to 1 hr.

4. In *Armadillidium* and *Porcellio* the pleopodal area loses water some ten to twenty times faster per unit area than the dorsal or ventral surface (some five times only in *Ligia*), but the absolute amount of water lost per unit time by the pleopods is less than that from either of the other areas mentioned.

5. Tables and graphs are given showing the rate of loss of water in mg./cm.^{2}/hr. in dry air at various temperatures for each species studied, both for 15 min. and 1 hr. exposures. These show a linear relationship between rate of evaporation and saturation deficit except for *Armadillidium* and *Glomeris* above 40° C. (where the

rate falls off), and *Ligia*. Reasons for these exceptions are suggested: curling in the first two, absolute size in the last.

6. The animals studied can be placed in a series as regards rate of loss of water, from *Ligia* (which shows the greatest rate) through *Philoscia*, *Oniscus*, *Porcellio*, *Cyclisticus* and *Armadillidium nasatum* to *A. vulgare*, which shows the lowest rate of loss. *Oniscus*, *Porcellio* and *Cylisticus* do not differ very greatly from one another in this respect. *Glomeris* loses water less rapidly than the majority of species of woodlice if exposed for 15 min., but, because the average rate of loss from woodlice falls if they are exposed for an hour, *Glomeris* appears to lose more rapidly than they do for an hour's exposure.

7. The highest temperature tolerated by woodlice for short exposures (15 min. or 1 hr.) does not vary greatly with humidity—temperatures are somewhat above 40° C. for 15 min. exposures—it is slightly higher at 50% R.H., due to a cooling effect, than in dry or saturated air; but for 24 hr. exposures, the animals die of desiccation and consequently the highest tolerable temperature is very much lower in dry air than in moist. These findings are also true, but to a less marked extent, of *Glomeris*.

8. The species can be arranged in a series as regards temperature tolerance which corresponds with the rate of evaporation series, *A. vulgare* having the highest temperature tolerance, *Ligia* the lowest.

9. After exposure to desiccation for 45 min. in dry air, or for 23 hr. in 50% R.H., both at room temperature, no species could live or recover weight if subsequently kept at 95% R.H. *Armadillidium* (but no others) could live and recover weight by absorption of water vapour if kept at 98% R.H., and all species could live and recover weight if kept in saturated air. Only living animals recovered weight by absorption of water vapour or liquid water. Animals which died after a period in the 'recovery' dishes always lost water very rapidly before doing so. The power to recover water from air at 98% R.H., possessed by *Armadillidium*, is limited by relative humidity not by saturation deficit.

10. The above results are discussed and the need for microclimatic data is stressed.

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REFERENCES

Allee, W. C. (1926). *J. Exp. Zool.* **45**, 255.
Beament, J. W. L. (1944). *J. Exp. Biol.* **21**, 115.
Cole, L. C. (1946). *Ecol. Monogr.* **16**, 50.
Edney, E. B. (1947). *Bull. Ent. Res.* **38**, 263.
Gunn, D. L. (1937). *J. Exp. Biol.* **14**, 178.
Hatchett, S. P. (1947). *Ecol. Monogr.* **17**, 47.
Heeley, W. (1941). *Proc. Zool. Soc. Lond.* B, **111**, 79.
Koidsumi, K. (1934). *Mem. Fac. Sci. Agric. Taihoku*, **12**, 1.
Lafon, M. (1943). *Ann. Sci. Nat.*, Ser. 11, **5**, 113.
Lafon, M. (1948). *Bull. Inst. Océanogr. Monaco*, **45**, no. 939, 1.
Lees, A. D. (1946). *Parasitology*, **37**, 1.
Lees, A. D. (1947). *J. Exp. Biol.* **23**, 379.
Lees, A. D. & Beament, J. W. L. (1948). *Quart. J. Micr. Sci.* **89**, 291.
Ludwig, D. (1937). *Physiol. Zool.* **10**, 342.
Mellanby, K. (1932). *J. Exp. Biol.* **9**, 222.
Mellanby, K. (1934). *Proc. Roy. Soc.* B, **116**, 139.
Mellanby, K. (1935). *Biol. Rev.* **10**, 317.
Miller, M. A. (1938). *Univ. Calif. Publ. Zool.* **43**, 113.
Numanoi, H. (1934). *J. Fac. Sci. Tokyo Univ.* **3**, 343.
Ramsay, J. A. (1935). *J. Exp. Biol.* **12**, 355.
Vandel, A. (1943). *Bull. biol.* Suppl. **30**, 1.
Verhoeff, K. W. (1920). *Z. wiss. Zool.* **118**, 365.
Waloff, N. (1941). *J. Exp. Biol.* **18**, 115.
Webb, W. M. & Sillem, C. (1906). *The British Woodlice.* London.
Wigglesworth, V. B. (1945). *J. Exp. Biol.* **21**, 97.
Wigglesworth, V. B. (1946). *Experientia*, **2**, 210.
Wigglesworth, V. B. (1948). *Biol. Rev.* **23**, 408.
Wigglesworth, V. B. & Gillett, J. D. (1936). *Proc. R. Ent. Soc. Lond.* A. **11**, 104.

LIPIDS OF THE NERVOUS SYSTEM OF THE SQUID *LOLIGO PEALII*

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Previous studies from this laboratory have indicated that the distribution of lipids in the mammalian central nervous system is very similar for a number of different species (Johnson, McNabb & Rossiter, 1948*a*). The same is true for the lipids of peripheral nerve (Johnson *et al.* 1948*b*). Johnson *et al.* (1949*a*, *b*) concluded that free cholesterol, cerebroside and sphingomyelin are the principal lipids of the myelin sheath of a mammalian nerve fibre, although the recent work of Brante (1949) suggests that one of the components of brain kephalin, phosphatidyl serine, may also be present in considerable quantities in the myelin sheath. These lipids have been called the myelin lipids.

Except for the honey-bee, which was studied by Patterson, Dumm & Richards (1945), little is known of the chemical nature of the lipids of invertebrate nervous systems. Since the squid has been used widely in physiological experiments (see, for example, Pumphrey & Young, 1938; Hodgkin & Huxley, 1939; Pumphrey, Schmitt & Young, 1940; Schmitt & Schmitt, 1940; Webb & Young, 1940; Steinbach, 1940; Cole, 1941; Curtis & Cole, 1942), it was decided to investigate this species further. The squid has the added advantage that both the anatomy (Young, 1932, 1936*a*–*c*, 1939) and the physiology (Prosser & Young, 1937; Young, 1938) of the nervous system have been extensively studied.

Bear, Schmitt & Young (1937*a*) concluded that, in addition to a connective tissue investment, the giant axon of the stellar nerve of the squid is surrounded by a lipid-containing sheath, in many respects similar to the myelin sheath of a vertebrate nerve and possessing a similar ultrastructure. Young (1935, 1936*a*) showed that the axoplasm of the giant fibre can readily be extruded. It seemed possible that a chemical study of the lipids of the nervous system of the squid and of the extruded axoplasm might shed some light on the nature of the lipids in this lipid-containing sheath of the giant axon and, indirectly, help in the understanding of the wider problem in the chemical nature of the 'myelin' of the myelin sheath of vertebrate nerve fibres.

METHODS

The common squid, *Loligo pealii*, was used in this study. The material was collected fresh by one of us (J. D. McC.) at the Marine Biological Laboratory, Woods Hole, during the summer of 1949. The required tissue was dissected from the nervous system of the squid, rapidly weighed, and placed in ethanol for despatch to London, Canada, where the analyses were performed.

The following samples were taken:

(1) *Central ganglia.* The samples included the entire circum-oesophageal ring, which Williams (1909) described as consisting of cerebral, pleural, pedal and visceral ganglia, together with the optic ganglia. Each sample consisted of the tissue from one squid.

(2) *Pallial nerve.* This is the connexion between the central ganglia and the stellate ganglion (Young, 1936*a*, *b*, 1939) and is sometimes referred to as the mantle connective nerve (Young, 1936*b*). Each sample contained tissue from two to four squids.

(3) *Entire giant fibre of stellar nerve.* The giant fibre in the hindmost stellar nerve of both sides was dissected as free as possible from the surrounding smaller fibres and connective tissue. Each sample contained tissue from eleven squids.

(4) *Axoplasm from giant axon.* The axoplasm was rolled out of the giant fibre using a modification of the technique described by Steinbach (1941). Since we were more concerned with obtaining axoplasm uncontaminated with sheath elements, no attempt was made to express all the axoplasm from each fibre. Analyses were not done on the fibre after the axoplasm had been expressed. This tissue contained much residual axoplasm and also much connective tissue (Bear *et al.* 1937*a*). Each sample contained tissue from fifteen squids.

Analytical methods

The lipids were extracted and estimated by the methods described by Johnson *et al.* (1948*a*, 1949*b*). The concentration of total phospholipin, free and total cholesterol, cerebroside (glycosphingoside), monoaminophospholipin (phosphoglyceride) and lecithin (phosphatidyl choline) was determined in each specimen, and from these figures was calculated the concentration of ester cholesterol, sphingomyelin (phosphosphingoside) and kephalin. Except where otherwise stated, the analyses were done in duplicate.

Accuracy of the methods

The accuracy of the methods is discussed in the paper by Johnson *et al.* (1949*b*). The coefficient of variation of replicate analyses on the same sample was found to be: cerebroside ± 3·5 %, free cholesterol ± 0·9 %, total cholesterol ± 0·9 %, total phospholipin ± 1·3 %, monoaminophospholipid ± 1·6 % and lecithin ± 5·1 %. The accuracy of the methods for those lipids derived by difference would usually be less. For the central ganglia and pallial nerve, where the mean of duplicate estimations is reported, the probable error of each determination would be less than the above figures. For the giant fibre of the stellar nerve and for the axoplasm, less tissue was available and duplicate analyses were not possible. For these tissues the value reported for sphingomyelin, derived by difference from single estimations, is little better than an approximation.

Specificity of the methods

The specificity of the methods has been discussed by Johnson *et al.* (1949*b*). In general, the methods are reasonably specific for the substances for which they were designed. Since little work has been done on the isolation and characterization of lipids from the nervous system of the squid, it is possible that unidentified lipids of properties similar to those occurring in the mammalian nervous systems could be responsible for some of the values reported. However, in the absence of evidence that the nervous system of invertebrates contains lipids other than those of the vertebrate nervous system (Patterson *et al.* 1945), it is convenient to retain, with the above reservations in mind, the usual lipid nomenclature.

RESULTS

Central ganglia. Table 1 gives the concentration of total phospholipin, total, free and ester cholesterol and cerebroside in the central ganglia of the squid. Table 2 gives the values for each of the individual phospholipins, i.e. sphingomyelin, lecithin and kephalin.

Table 1. *Lipids of the central ganglia of squid* (*mg. per* 100 *mg. fresh tissue*)

	Total phospholipin (1)	Total cholesterol (2)	Free cholesterol (3)	Ester cholesterol (2) − (3)	Cerebroside (4)
	3·11	0·73	0·72	0·01	0·06
	3·15	0·74	0·73	0·01	0·04
	3·30	0·74	0·73	0·01	0
	3·27	0·69	0·69	0·00	0
Mean (±σ)	3·21 (±0·09)	0·73 (±0·06)	0·72 (±0·05)	0·01 (±0·05)	0·03 (±0·08)

Table 2. *Phospholipins of the central ganglia of squid* (*mg. per* 100 *mg. fresh tissue*)

	Total phospholipin (1)	Monoamino-phospholipin (5)	Sphingomyelin (1) − (5)	Lecithin (6)	Kephalin (5) − (6)
	3·11	2·83	0·28	1·07	1·76
	3·15	2·72	0·43	1·27	1·45
	3·30	2·88	0·42	1·23	1·65
	3·27	2·82	0·45	1·11	1·71
Mean (±σ)	3·21 (±0·09)	2·81 (±0·07)	0·40 (±0·06)	1·17 (±0·08)	1·64 (±0·11)

These figures are all in terms of mg./100 mg. fresh tissue. Since the wet weight to dry weight ratio was not greatly different from that previously found for the whole brain of the guinea-pig, cat and rabbit, the figures of Tables 1 and 2 can be compared with those reported by Johnson *et al.* (1948*a*) for these species. The concentration of total phospholipin was of the same order as that in mammalian brain, while the concentration of the cholesterol was much less. As was found for the mammals, only a negligible proportion of the cholesterol was in the ester form. A notable difference from the mammalian brain was the absence of cerebroside. The concentration of lecithin and kephalin was of the same order as that in the mammalian brain, but the concentration of sphingomyelin was considerably less.

Table 3. *Lipids of the pallial nerve of the squid* (*mg. per* 100 *mg. fresh tissue*)

Lipids	Sample 1	Sample 2	Mean
Total phospholipin (1)	0·87	1·08	0·98
Total cholesterol (2)	0·29	0·33	0·31
Free cholesterol (3)	0·28	0·32	0·30
Ester cholesterol (2) – (3)	0·01	0·01	0·01
Cerebroside (4)	0·00	0·05	0·03
Monoaminophospholipin (5)	0·62	0·78	0·70
Sphingomyelin (1) – (5)	0·25	0·30	0·28
Lecithin (6)	0·36	0·48	0·42
Kephalin (5) – (6)	0·26	0·30	0·28

Pallial nerve. Table 3 gives the concentration of the same lipids in two samples of pallial nerve. In terms of wet weight of tissue the concentration of each of the lipids in the pallial nerve was much less than that of the central ganglia. This difference can only to a slight degree be explained by a difference in water content. It may possibly be associated with the larger amount of connective tissue, presumably containing little lipid, in the pallial nerve. As in the central ganglia, there was little ester cholesterol or cerebroside. In each of the four samples of central ganglia the concentration of kephalin exceeded that of lecithin, whereas in each of the two samples of pallial nerve the concentration of lecithin exceeded that of kephalin. Sphingomyelin accounted for a higher percentage of the total phospholipin in pallial nerve.

Table 4. *Lipids of the stellar nerve of squid* (*mg. per* 100 *mg. fresh tissue*)

Lipids	Whole giant fibre			Axoplasm			Axon sheaths (calculated)
	Sample 1	Sample 2	Mean	Sample 1	Sample 2	Mean	
Total phospholipin	0·332	0·335	0·334	0·185	0·174	0·180	1·03
Total cholesterol	0·089	0·078	0·084	0·029	0·039	0·034	0·31
Monoaminophospholipin	0·282	0·238	0·060	0·146	0·160	0·153	0·74
Sphingomyelin	0·050	0·097	0·074	0·039	0·014	0·027	0·29

Stellar nerve. Table 4 gives the concentration of lipids in the whole fibre of the stellar nerve and also that of the lipids in the extruded axoplasm. On a wet weight basis the concentration of each of the lipids was less than that in the pallial nerve and considerably less than that in the central ganglia. As before, no ester cholesterol or cerebroside was detected, but these negative readings were somewhat equivocal, since the size of the sample was so small that the analytical methods were pushed to their extreme limits. However, the absence of these two lipids from both the central ganglia and the pallial nerve, where larger tissue samples were available, would make it probable that these lipids were also absent from the stellar nerve.

The concentration of each of the lipids was greater in the whole fibre than in the axoplasm. There can be little doubt that these lipids are concentrated in the sheaths surrounding the axons. The last column of Table 4 gives the concentration of lipids in the axon sheaths calculated from the concentration of lipids in the whole fibre and in the axoplasm, based on the report of Bear & Schmitt (1939) that the sheaths represent 18 % of the wet weight of the fibre. The concentration of lipids in the lipid-containing sheath itself would be many times the values calculated for the total axon sheaths for, as can be seen from fig. 2 of the paper by Bear *et al.* (1937*a*), the axon sheaths are comprised chiefly of connective tissue, the lipid-containing sheath representing only a small fraction of the total.

If it is assumed that the connective tissue sheaths are free from lipid, the figures for the axon sheaths, while giving no information about the absolute concentration of lipids in the lipid-containing sheath, give an indication of their relative distribution. The figures for total phospholipin, total cholesterol and monoaminophospholipin are reasonably accurate but, as has been pointed out already, the accuracy of the figures for sphingomyelin, calculated from figures that are themselves obtained by difference, is considerably less.

DISCUSSION

Cerebroside in invertebrate nervous systems. The absence of cerebroside from the nervous system of the squid is in accordance with the earlier observation of Lanfranchi (1938) that there is no cerebroside in the nervous system of the octopus. Patterson *et al.* (1945) found no cerebroside in the nervous system of the honey-bee. We have also found no cerebroside in the nervous system of the arachnoid crab, *Limulus*. This might suggest that the nervous systems of all invertebrates do not contain cerebroside, but subsequent analyses have shown that the ventral nerve cord of the crustacean, *Libinia*, contains 0·64 ± 0·11 mg. per 100 g. fresh tissue of a substance that estimates as cerebroside, while Brante (1949) reported the presence of 0·8 mg. cerebroside per 100 g. dry tissue in the claw nerve of the lobster.

The presence of cerebroside in the nervous system of vertebrates appears to be the rule. Lanfranchi (1938) found cerebroside in the brain of a number of vertebrate species, while, in experiments yet to be reported, we have invariably found cerebroside, although in widely differing concentrations, in the brains and spinal cords of marine and fresh-water elasmobranch and teleost fish, amphibians, reptiles, birds and mammals.

Lipids of the axoplasm. Our analyses would suggest that there is a small, though definite, concentration of lipid in the axoplasm of squid nerve. It is unlikely that this small concentration of lipid is the result of contamination of the axoplasm with sheath tissue, since the relative distribution of the lipids in the axoplasm was different from that of the whole nerve fibre, e.g. a lower concentration of cholesterol relative to total phospholipin. If the axoplasm were really free from lipid and the lipid estimated was a contaminant from the sheath, one would expect to find the same relative concentration of each of the lipids in the axoplasm as was found in the whole fibre. Also Boell & Nachmansohn (1940) found little cholinesterase in axoplasm obtained by a similar method, although high concentrations were found in the sheaths.

If lipid is present in the axoplasm of squid nerve, it is presumably also present in the axoplasm of vertebrate nerves. The concentration of lipids in the axoplasm, approximately 0·25 mg. per 100 mg. wet tissue, is of a different order from the concentration of lipids, estimated to be between 30 and 50 mg. per 100 mg. wet tissue, in the myelin sheath of vertebrate nerve fibres (Schmitt, Bear and Palmer, 1941; Brante, 1949; Engström & Lüthy, 1950). Engström & Lüthy (1950) measured the ability of the sciatic nerve of the frog to absorb X-rays, both before and after extraction with alcohol-ether and petroleum-ether, and concluded that there is no lipid in the axoplasm. It is doubtful, however, if their methods would be able to detect a concentration of lipid as small as that found in the squid axoplasm.

Although the proteins of the axoplasm of the giant axon have been studied by Bear *et al.* (1937*c*) and a number of workers have investigated the electrolytes (Bear & Schmitt, 1939; Webb & Young, 1940; Steinbach, 1941; Steinbach & Spiegelman, 1943), we are aware of no chemical determinations of the lipids of squid axoplasm.

Analysis of the form birefringence of the axon of the giant fibre led Bear *et al.* (1937*b*) to conclude that a small fraction of the axon consists of highly asymmetric particles orientated parallel with the long axis of the fibre. Using the electron microscope, Richards, Steinbach & Anderson (1943) have described particulate structures in the axoplasm of the giant axon of the squid, and Schmitt & Geren (1950) and Schmitt (1950) have described filamentous structures in the axon of nerves of the squid, frog, rat and man. Since the submicroscopic particles ('microsomes') of the cytoplasm of a variety of cells such as liver (Claude, 1946), lymphosarcoma (Claude, 1944), pancreas and embryonic tissue are rich in lipid, chiefly phospholipin, it is possible that the lipid of the axoplasm is associated with the organized particles demonstrated by the specialized techniques of polarization optics and electron microscopy.

Distribution of lipids in the nervous system of the squid. The distribution of lipids in the nervous system of the squid is different from that in the mammalian nervous system. The absence of cerebroside has already been mentioned. Patterson *et al.* (1945) pointed out that the lipids of the nervous system of the honey-bee were similar to those of the vertebrate at an age before myelination had commenced.

In Table 5 the lipids of the nervous system of the squid are given relative to the concentration of total phospholipin, which was represented as 100. The table also gives the figures for both grey matter and white matter of infant brain and adult brain reported by Johnson *et al.* (1949*a*) and the figures for the sciatic nerve of the cat reported by Johnson *et al.* (1949*b*). This method of presenting the data is entirely arbitrary, but it readily enables the lipids of one species to be compared with those of another, is independent of the water content of the tissues, and has the added advantage that the individual phospholipins are expressed as a percentage of total phospholipin.

Table 5. *Distribution of lipids of squid nervous system compared with that in the nervous system of mammals. All figures are relative to total phospholipin, arbitrarily represented as* 100

Lipid	Squid					Man				Cat
	Pallial nerve	Whole fibre (stellar nerve)	Sheath giant fibre (calc.)	Axopl. giant fibre (stellar nerve)	Central ganglia	Infant grey matter	Infant white matter	Adult grey matter	Adult white matter	Sciat whit
	Group I			Group II		Group III			Group IV	
Total phospholipin	100	100	100	100	100	100	100	100	100	100
Total cholesterol	32	25	30	19	23	26	32	29	60	53
Cerebroside	0	0	0	0	0	28	28	26	68	40
Monoamino-phospholipin	71	78	72	85	88	93	94	84	71	61
Sphingomyelin	29	22	28	15	12	7	6	16	29	39
Lecithin	43	—	—	—	37	40	41	29	19	12
Kephalin	29	—	—	—	51	53	53	55	52	49

The lipids of the tissues represented in Table 5 appear to divide themselves into four groups:

Group I. This group contains the pallial nerve and the sheath tissue of the giant fibre of the stellar nerve of the squid. In both these tissues the proportion of sheath tissue to axoplasm would be high (Bear *et al.* 1937*a*, fig. 2; Young, 1939, fig. 38).

Group II. This group is represented by the central ganglia of the squid and the axoplasm of the giant fibre. Tissues of this group are distinguished from those of group I by containing relatively less total cholesterol and sphingomyelin. The whole giant fibre of the stellar nerve is somewhat intermediate between group I and group II.

Another consistent difference between group I and group II is that the pallial nerve contains more lecithin than kephalin, whereas in the central ganglia of the squid kephalin exceeded lecithin. If it is conceded that the tissues of group I contain more sheath elements than those of group II, these findings would suggest that the lipid-containing sheath of the squid nerve is rich in cholesterol, sphingomyelin and, perhaps, lecithin. It is interesting to note that Brante (1949) reported a high concentration of lecithin in the claw nerve of the lobster, and we have found that in the ventral nerve cord of both *Libinia* and *Limulus* the concentration of lecithin invariably exceeded that of kephalin.

Group III. This group of tissues includes both grey matter and white matter of infant brain and the grey matter of adult brain, tissues poor in myelinated fibres. With the exception of cerebroside, the distribution of lipids is extremely similar to that of the squid tissues of group II. This similarity between the distribution of lipids of invertebrate nervous systems to that of the grey matter of mammalian brain and to that of the brain of newborn infants has also been found for the ventral nerve cord of *Limulus* and *Libinia*.

Group IV. The tissues of this group include the white matter of adult brain and the sciatic nerve of the cat, tissues rich in myelinated nerve fibres. These tissues contain relatively more of the myelin lipids, i.e. cholesterol, cerebroside and sphingomyelin.

Polarization optics and X-ray diffraction studies have suggested that all nerve axons are surrounded by a lipid-containing sheath and that there is an essential unity in the ultrastructure of the sheath, whether the nerve be vertebrate or invertebrate, myelinated or unmyelinated (see Schmitt, 1936; Schmitt & Bear, 1939; Schmitt *et al.* 1941 for references). The work of Bear *et al.* (1937*a*) would indicate that the squid is no exception. Our data are in every way consistent with the view that the giant axon of the stellar nerve of the squid possesses a lipid-containing sheath. However, despite a similarity in ultrastructure, the lipid components of the sheath would appear to differ in a number of respects from those of mammalian nerve.

SUMMARY

1. The concentration of total phospholipin, free and total cholesterol, cerebroside (glycosphingoside), monoaminophospholipin (phosphoglyceride) and lecithin (phosphatidyl choline) was determined in the central ganglia, pallial nerve, whole giant fibre of the stellar nerve, and axoplasm of the giant fibre of the squid, *Loligo pealii*. From these figures was calculated the concentration of ester cholesterol, sphingomyelin (phosphosphingoside) and kephalin.

2. The concentration of total phospholipin in the central ganglia was of the same order as that found in mammalian brain, but the concentration of cholesterol and sphingomyelin was less.

3. The concentration of lipids in the pallial nerve was less than that in the central ganglia. The concentration of lecithin exceeded that of kephalin, while sphingomyelin accounted for higher percentage for phospholipin than in the central ganglia.

4. The concentration of lipids in the whole giant fibre of the stellar nerve, less than that in either the central ganglia or in the pallial nerve, was greater than that in the axoplasm.

5. The results are consistent with the view that the axon of the giant fibre of the stellar nerve is surrounded by a thin lipid-containing sheath.

6. Neither cerebroside nor ester cholesterol was found in the nervous system of the squid.

7. With the exception of cerebroside, the relative concentration of lipids in the central ganglia and in the axoplasm of the giant fibre resembled that in parts of the nervous system of man that contain relatively few myelinated fibres, i.e. the grey matter of the brain of the adult or either the grey matter or the white matter of the brain of the newborn infant.

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REFERENCES

Bear, R. S. & Schmitt, F. O. (1939). *J. Cell. Comp. Physiol.* **14**, 205.
Bear, R. S., Schmitt, F. O. & Young, J. Z. (1937*a*). *Proc. Roy. Soc.* B, **123**, 496.
Bear, R. S., Schmitt, F. O. & Young, J. Z. (1937*b*). *Proc. Roy. Soc.* B, **123**, 505.
Bear, R. S., Schmitt, F. O. & Young, J. Z. (1937*c*). *Proc. Roy. Soc.* B, **123**, 520.
Boell, E. J. & Nachmansohn, D. (1940). *Science*, **92**, 513.
Brante, G. (1949). *Acta physiol. scand.* **18**, 1, Suppl. 63.
Claude, A. (1944). *J. Exp. Med.* **80**, 19.
Claude, A. (1946). *J. Exp. Med.* **84**, 61.
Cole, K. S. (1941). *J. Gen. Physiol.* **25**, 29.
Curtis, H. J. & Cole, K. S. (1942). *J. Cell. Comp. Physiol.* **19**, 135.
Engström, A. & Lüthy, H. (1950). *Exp. Cell. Res.* **1**, 81.
Hodgkin, A. L. & Huxley, A. F. (1939). *Nature, Lond.*, **144**, 710.
Johnson, A. C., McNabb, A. R. & Rossiter, R. J. (1948*a*). *Biochem. J.* **43**, 573.
Johnson, A. C., McNabb, A. R. & Rossiter, R. J. (1948*b*). *Biochem. J.* **43**, 578.
Johnson, A. C., McNabb, A. R. & Rossiter, R. J. (1949*a*). *Biochem. J.* **44**, 494.
Johnson, A. C., McNabb, A. R. & Rossiter, R. J. (1949*b*). *Biochem. J.* **45**, 500.
Lanfranchi, E. (1938). *Arch. sci. biol.* **24**, 120.
Patterson, E. K., Dumm, M. E. & Richards, A. G. (1945). *Arch. Biochem.* **7**, 201.
Prosser, C. L. & Young, J. Z. (1937). *Biol. Bull. Woods Hole*, **73**, 237.
Pumphrey, R. J., Schmitt, O. H. & Young, J. Z. (1940). *J. Physiol.* **98**, 47.
Pumphrey, R. J. & Young, J. Z. (1938). *J. Exp. Biol.* **15**, 453.
Richards, A. G., Steinbach, H. B. & Anderson, T. F. (1943). *J. Cell. Comp. Physiol.* **21**, 129.
Schmitt, F. O. (1936). *Symp. Quant. Biol.* **4**, 7.
Schmitt, F. O. (1950). *J. Exp. Zool.* (in the Press).
Schmitt, F. O. & Bear, R. S. (1939). *Biol. Rev.* **14**, 27.
Schmitt, F. O., Bear, R. S. & Palmer, K. J. (1941). *J. Cell. Comp. Physiol.* **18**, 31.
Schmitt, F. O. & Geren, B. B. (1950). *J. Exp. Med.* **91**, 499.
Schmitt, F. O. & Schmitt, O. H. (1940). *J. Physiol.* **98**, 26.
Steinbach, H. B. (1940). *J. Cell. Comp. Physiol.* **15**, 373.
Steinbach, H. B. (1941). *J. Cell. Comp. Physiol.* **17**, 57.
Steinbach, H. B. & Spiegelman, S. (1943). *J. Cell. Comp. Physiol.* **22**, 187.
Webb, D. A. & Young, J. Z. (1940). *J. Physiol.* **98**, 299.
Williams, L. W. (1909). *The Anatomy of the Common Squid*, Loligo pealii (*Leseur*). Leiden.
Young, J. Z. (1932). *Quart. J. Micr. Sci.* **75**, 1.
Young, J. Z. (1935). *J. Physiol.* **83**, 27*P*.
Young, J. Z. (1936*a*). *Proc. Roy. Soc.* B, **121**, 319.
Young, J. Z. (1936*b*). *Symp. Quant. Biol.* **4**, 1.
Young, J. Z. (1936*c*). *Quant. J. Micr. Sci.* **79**, 367.
Young, J. Z. (1938). *J. Exp. Biol.* **15**, 170.
Young, J. Z. (1939). *Philos. Trans.* B, **229**, 465.